FANCM, the mouse that roared

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FANCM, the mouse that roared

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Chance favors the optimistic mind.

Sietske Bakker, after Louis Pasteur

This thesis is dedicated to all FA patients and their families and loved ones.

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Contents

Chapter 1

Introduction

Fanconi anemia

Fanconi anemia (FA) is a rare autosomal recessive or X-linked inherited type of anemia as a consequence of bone marrow failure. The disorder was named after Guido Fanconi (1892-1979) since he was the first to describe its symptoms in a case report published in 1927[1]. He reported a family of five children of which three boys developed anemia between the ages of five and seven and died. The brothers also had physical abnormalities such as a small head, skin pigmentation, cutaneous haemorrhage, and underdevelopment of the testes [1,2]. In retrospect these three brothers were the first FA patients documented and their symptoms are still hallmarks of FA.

Fanconi anemia - Clinical Symptoms

The median age of diagnosis for FA is 7 years [3]. Usually hematological problems are the first indication for FA, but the disease is also characterized by a broad range of developmental abnormalities [3]. Patients can present with congenital malformations of the head (microcephaly), eyes (microphthalmia), skeleton (thumb and or radius), and an overall short stature and low birth weight. Genital malformations are also common in FA patients as well as hypogonadism and infertility, although some FA patients have got children. Less common symptoms include gastrointestinal defects, cardiac defects, central nervous system defects, and neural tube defects [3]. Of all FA patients around one third has a normal physical development [4].

Although FA is a genetic disease there is much phenotypic and pathogenic variability, even between related patients with an identical mutation. This is illustrated by the variation in clinical symptoms in four FA patients from two related consanguineous families who had an identical FA mutation [5]. This demonstrates that a different environment combined with genetic variation can all contribute to the penetrance of FA associated phenotypes.

Fanconi anemia - Diagnosis

The variability of the clinical symptoms associated with FA makes it difficult to diagnose the disease. Therefore, a more uniform test has been developed to diagnose FA [6]. This test exploits the clastogenic (chromosome-breaking) response of cells from FA patients to treatment with DNA crosslinking agents such as mitomycin C (MMC) or diepoxybutane (DEB). These compounds induce interstrand crosslinks (ICLs) in the DNA. After treatment with crosslinking agents, FA cells show an increased number of chromosome breaks as can be evaluated in a metaphase spread. This chromosomal breakage test is now the standard test to diagnose FA. If the specific mutation is known within an FA family, genetic testing can also be used for diagnosis. Figure 1.1 shows a metaphase spread of FA deficient mouse embryonic fibroblasts (MEFs) after MMC treatment and the collection of chromosomal aberrations frequently observed in FA cells.

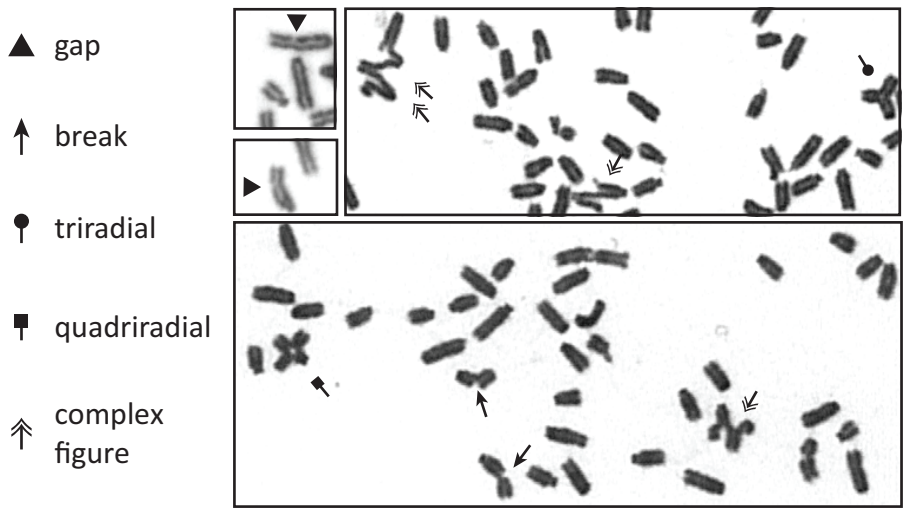

FIGURE 1.1
Picture of metaphase spreads of *Fancm*-deficient mouse embryonic fibroblasts (MEFs) after MMC treatment with a collection of different chromosomal aberrations typical for FA.

Fanconi anemia - Bone marrow failure

The development of anemia as a consequence of bone marrow failure (BMF) is the symptom of FA that requires treatment most frequently and it remains the primary cause of death for FA patients. By age 40, 90% of FA patients will have developed bone marrow failure [7,8]. The anemia can initially be treated by hematopoietic growth factors or androgen therapy to improve blood counts, however, all patients eventually become insensitive to this treatment and rely on blood transfusions to maintain adequate blood counts [3]. A more definitive therapy is hematopoietic stem cell transplantation (HSCT) to repopulate the bone marrow. To date, the 5-year survival frequency after transplantation with a human leukocyte antigen (HLA)-matched sibling donor is approximately 85% [9]. The survival frequency for transplantation with an unrelated donor drops to 52% at 3 years [9,10].

Since an HLA-matched donor is not always available, the possibility of gene therapy has received much attention in the FA field. Gene therapy involves the correction of the FA defect in HSC by viral delivery of wild-type cDNA of the affected gene and the reintroduction of these corrected cells in the bone marrow. The corrected HSC would subsequently compete out the affected FA-HSC due to a growth advantage and could repopulate the bone marrow of an FA patient. The feasibility of this approach has already been demonstrated by nature itself as some patients show somatic mosaicism indicating a selective advantage of spontaneously corrected HSC [11]. It was even suggested that a single prenatally corrected HSC of one twin suffering from FA was capable of repopulating the bone marrow of both children and preventing the onset of hematological problems [12].

Gene therapy, however, requires the use of retroviral constructs which pose the threat of oncogenesis as a consequence of random viral integration. An alternative approach could be the use of somatic cells from FA patients to be reprogrammed to pluripotency to generate patient-specific induced pluripotent stem cells (iPS) cells. It has already been shown that somatic FA cells could be reprogrammed after correction of the FA defect. Furthermore, these corrected FA-iPS cells could give rise to haematopoietic progenitors of the myeloid and erythroid lineages that were phenotypically normal [13]. These cells could in principle be used for HSCT as the ideal HLA-matched donor.

Fanconi anemia - Cancer

The median age at death for FA patients was only 19 years in the nineties of the 20th century but has increased to 30 years in 2000, especially due to the improved success rate of HSCT [4,10]. However, the overall life expectancy of FA patients is still severely reduced due to an increased risk to develop cancer. Especially the risk to develop myelodysplasia or acute myeloid leukemia (AML) is increased, with an AML incidence of 33% by age 40 posing a more than 1000-fold increased incidence compared to the normal population [7,8]. FA patients are also at risk to develop solid tumors especially squamous cell carcinoma (SCC) of the head and neck (500-fold increased risk), SCC of the esophagus (1000-fold increased risk), and gynecologic cancers [7,8,14]. Unfortunately, the treatment of leukemia and solid tumors in FA patients is complicated by the severe side effects of conventional chemotherapy due to defects in DNA repair in normal tissues of FA patients, as will be discussed in chapter 7.

Fanconi anemia - Genetics

FA is not only clinically but also genetically a heterogeneous disorder. The hypersensitivity of FA cells to MMC or DEB has made it possible to classify FA in different complementation groups. The assignment of a new FA complementation group is as follows: cells from an FA patient for which the mutated gene is unknown are fused to cells from a known complementation group. This results in a hybrid cell line with a 4N DNA content, which is tested for ICL (MMC or DEB) sensitivity. If the hybrid cell line is ICL sensitive, the two cell types are not able to complement each other and they have a defect in the same gene. If the hybrid cell line has become ICL resistant, the two cell types had a different genetic defect and belong to a separate complementation group [15]. This method, however, can result in a wrong assignment due to spontaneous phenotypic reversion, the process by which a wild-type allele can be recreated due to gene conversion or intragenic recombination [16] Therefore, a new complementation group should be based on at least two patients, or, if only one patient cell line is available, on a new complementing gene with pathogenic mutations [16]. By applying this complementation analysis several different complementation groups have been determined and 15 mutated genes (*FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN/PALB2, FANCO/Rad51C* and *FANCP*) have been identified [17-20]. Some patients do not have mutations in any of these genes and belong to unclassified complementation groups.

So it is expected that additional FA genes will be discovered in the future [21].

Fanconi anemia - FA genes involved in cancer susceptibility syndromes

The discovery that *FANCD1* was identical to the breast cancer susceptibility gene 2 (*BRCA2*) [22] was an unexpected finding that has generated much interest into the involvement of FA genes in cancer [23]. Heterozygous carriers of *FANCD1/BRCA2* mutations are highly susceptible to breast, ovarian and pancreas tumors, which arise after loss of the wild-type allele. *FANCJ*, *FANCN*, and *FANCO* are other cancer susceptibility genes which also give rise to FA [24-28]. *FANCJ* is also known as *BRIP1* or *BACH1* and interacts with *BRCA1*, another breast cancer susceptibility gene. *FANCN* interacts with *BRCA2* and is therefore more commonly referred to as *PALB2* (partner and localizer of *BRCA2*) [29]. *FANCN* mutations have been identified in patients with familial pancreatic cancer [28]. Heterozygous mutations in *FANCO* were found to confer an increased risk to breast and ovarian cancer [30,31]. To date, no increased cancer risk for FA heterozygotes that belong to FA core complex groups have been reported although there is some evidence that *FANCC* mutations are possibly low penetrance breast cancer susceptibility alleles [32].

Fanconi anemia - the FA pathway for ICL repair

FA cells are characterized by a hypersensitivity to ICL agents, which is manifested by increased chromosomal breakage, a prolonged G2 arrest, and reduced survival. Based on this shared phenotype, all FA proteins are thought to function together in the so-called FA pathway for ICL repair. ICL agents induce either intra- or interstrand crosslinks in the DNA of which the interstrand crosslink is considered the most cytotoxic, since it prevents strand segregation thereby blocking replication, transcription and chromatin maintenance [33]. ICLs therefore need to be removed and the integrity of the DNA restored in order for a cell to survive. ICL repair requires the coordination of multiple different repair pathways, which varies per cell cycle phase. The FA pathway is thought to play a critical role in the coordination of these different repair pathways and some FA proteins are thought to be also directly involved in the repair process itself. Chapter 7 contains a more extensive discussion of the molecular details of the FA pathway for ICL repair.

Fanconi anemia - acetaldehyde repair

FA cells are uniquely sensitive to compounds that induce ICLs such as MMC or DEB, however exposure to these compounds is limited and therefore unlikely to be the normal substrate for the FA pathway. A central question in the FA field has been what the endogenous ICL source is that necessitated the evolution of the FA pathway.

Formaldehyde is a potential candidate, since this molecule is generated during normal metabolism and known to induce DNA and DNA-protein crosslinks. Moreover, FA cells are hypersensitive to this agent [34]. In agreement with this notion recent exciting experiments with *Fancd2* mutant mice suggest that aldehydes could indeed

be endogenous substrates for the FA pathway [35]. It was demonstrated that in the absence of fetal and maternal ALDH2, an enzyme that detoxifies acetaldehyde, *Fancd2*-deficient mice were not viable. Moreover, double homozygous mutant animals born from a cross between heterozygous animals died within 3 to 6 months due to the development of acute lymphoblastic leukemia (ALL). In addition, when *Fancd2/Aldh2*-deficient embryos were exposed to ethanol *in utero* they developed severe physical abnormalities reminiscent of fetal alcohol syndrome and were underrepresented [35]. Also young *Fancd2/Aldh2*-deficient animals had a severe reduction in bone marrow cellularity and showed γH2AX induction after exposure to ethanol. These data collectively demonstrate that excess endogenous or exogenous acetaldehyde, due to a defect in detoxification, results in severe toxicity in the absence of FANCD2 [35].

It remains to be determined if this phenotype also extends to other FA mouse models. Mouse embryonic fibroblasts (MEFs) derived from other FA core complex animals were not sensitive to formaldehyde whereas *Fancd2*-deficient MEFs were [36]. However, FA core complex deficient splenocytes or DT40 cells were again sensitive to acetaldehyde [35]. In addition, DT40 cells mutant for TLS or HDR were not sensitive to acetaldehyde whereas these proteins are thought to play an important role in ICL repair (as will be discussed in chapter 7).

Outline of this thesis

This thesis contributes to the FA field by studying the pathology of FA in mouse models. First an extensive overview is given in chapter 2 on recent insights obtained by studying FA mouse models. In chapter 3 we describe the unique characteristics of the only patient belonging to the FA-M complementation group. In this chapter we demonstrate that this patient had additional mutations in another FA gene, *Fanca*, making it unclear how mutations in FANCM contribute to an FA phenotype. Therefore in chapter 4 we describe a mouse model which we have made by deleting exon 2 of *Fancm*. This mouse model showed several characteristic FA phenotypes such as crosslinker sensitivity, hypogonadism, and reduced FANCD2 monoubiquitination, but also several non-FA phenotypes. In chapter 5 another FA mouse model is introduced, the *Fancf* mouse model. Also *Fancf*-deficient mice showed the characteristic FA-associated phenotypes in mice. However, these mice were also prone to develop granulosa cell tumors of the ovary, a cancer type not previously associated with FA in mice. In chapter 6 the mouse models introduced in chapter 4 and 5 were crossed with the intestinal tumor mouse model $Apc^{Min/+}$. Paradoxically whereas *Fancf* deficiency did not alter the latency or severity of tumorigenesis in $Apc^{Min/+}$ mice, *Fancm* deficiency did. This could indicate that *Fancm* plays a role outside the FA pathway in intestinal tumor suppression. In the discussion (chapter 7) we will speculate about the role of FANCM and the other FA proteins in a new model for ICL repair. This alternative model for ICL repair is recombination dependent but DSB independent and aims to explain the unique characteristics associated with *Fancm*-deficiency that led to a mouse that roared.

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Chapter 2

Recent insights into the pathology of Fanconi anemia mouse models

Manuscript in preparation

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Abstract

Fanconi anemia (FA) is a rare autosomal recessive or X-linked inherited disease and mutations in at least 15 different genes have been identified that cause this disorder. FA patients are characterized by an increased incidence of bone marrow failure, hematological malignancies, and solid tumors. Cells from FA patients show a pronounced sensitivity to interstrand crosslinking agents (ICLs) and all FA gene products are thought to function together in the FA pathway that is essential for ICL repair. To study this disorder several FA mouse models have been generated. These mutant mice all show the characteristic ICL sensitivity, but differ in their predisposition to hematological problems or malignancies which are common in FA patients. This review extrapolates on this apparent paradox and summarizes the recent insight which FA mouse models have uncovered on the pathology of FA.

Introduction

Fanconi anemia patients suffer from bone marrow failure (BMF), developmental abnormalities, and an increased cancer risk. To date 15 genes have been identified which cause FA or an FA-like disorder [1-3]. Despite the genetic and phenotypic heterogeneity of FA, FA cells from all complementation groups share a characteristic hypersensitive to interstrand crosslinking agents (ICLs). The identification of the FA genes and analysis of the function of encoded proteins has uncovered a molecular pathway important for ICL resistance termed the FA pathway.

Most FA proteins are found in a complex called the FA core complex. This complex consists of 8 FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM), and four FA-Associated Proteins (FAAP24, FAAP100, MHF1 and MHF2) [4]. The formation of the FA core complex is necessary for the efficient monoubiquitination of FANCD2 and FANCI by the E3 ubiqutin ligase FANCL with UBE2T as E2 ligase [5] . The remaining FA proteins FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/RAD51C and FANCP/SLX4 act downstream or parallel to this monoubiquitination step in the FA pathway to facilitate ICL repair [6].

Following the identification of FA genes in humans, blast searches for orthologs in other species were undertaken. The conservation of most FA proteins, especially the FA core complex members, seems limited to vertebrates [7]. In *Drosophila melanogaster* or the urochordate *Ciona intestinalis* only orthologs of FANCD2, FANCL and FANCM exist, and it appears that FANCL by itself can monoubiquitinate FANCD2 and confer crosslinker resistance in the fly [8,9]. It remains enigmatic why there are so many FA core complex genes to regulate FANCL ubiquitin ligase activity, and the enigma is enhanced by the fact that most FA core complex members, apart from FANCL and FANCM, are orphan proteins lacking functional domains. Yet, in vertebrates the FA core complex has evolved and each member is essential for a functional FA pathway.

Anemia as a consequence of BMF is usually the first symptom of FA patients. In addition, more than two third of FA patients also present with a wide range of develop-

TABLE 2.1
Mouse models for Fanconi anemia and the associated gene disruptions

Gene	Target Disruption	Reference
Fanca	exons 4–7	[15]
Fanca	exons 1–6,	[16]
Fanca	exon 37	[17]
Fancc	exon 8	[18]
Fancc	exon 9	[19]
Fancd1	various	[20]
Fancd2	exons 26 and 27	[21]
Fancd2	gene trap insertion in intron 1	[33]
Fancf	exon 1	[22]
Fancg	exons 1–4	[34]
Fancg	exons 2-9	[24]
Fancl	exons 4–14	[25]
Fancm	Exon 2	[26]
Fancn	gene trap insertion in intron 1	[27,28]
Fanco	exons 2-3	[29]
Fancp	gene trap insertion in intron 2	[32]

mental abnormalities such as microcephaly, microphthalmia, abnormalities of the skeleton (thumb and/or radius), short stature and low birth weight, and genital malformations [10]. FA patients also have a high risk to develop cancer, especially acute myeloid leukemia (AML), squamous cell carcinoma (SCC) of the head and neck, SCC of the esophagus, liver tumors, and gynecologic cancers [11,12]. The cumulative probability in FA patients to develop leukemia, solid tumors, and liver tumors is almost 40% by age 30, about 50% by age 45, and 76% by age 45 [13].

To study the above mentioned FA phenotypes and to develop a potential treatment for FA associated symptoms the laboratory mouse (*Mus musculus*) is a frequently used model system. A number of advantages including the relative ease to work with mice as compared to other rodents, the extensive physiological and molecular similarities to humans [14], and the ability to perform gene targeting and disrupt FA genes have made mice the most frequent animal model to study FA.

FA mouse models

By disruption of FA genes several FA mouse models have been created to date: three for *Fanca* [15-17], two for *Fancc* [18,19] several for *Fancd1/Brca2* [20], one for *Fancd2* [21], one for *Fancf* [22], two for *Fancg* [23,24], one for *Fancl/POG* [25], one for *Fancm* [26], two for *Fancn/Palb2* [27,28], several for *Fanco/Rad51C* [29-31], and one for *Fan-*

cp/Slx4 [32]. Table 1 shows all FA mouse models published to date with their gene disruptions. Cells derived from these mutant mice were all sensitive to ICL-inducing agents, the characteristic cellular phenotype of FA.

Embryonic and perinatal lethality

Embryonic lethality due to homozygous disruption of FA genes appeared to be restricted to non-FA core complex genes such as *Fancd1/Brca2*, *Fancn/Palb2* and *Fanco/Rad51C*. An exception are the *Fancl* mice which are embryonic lethal on a pure 129/Sv or C57BL/6J background but viable on a mixed C57BL/6JxFVB background [25]. *Fancd1/Brca2* and *Fancn/Palb2* homozygous mice die early during embryonic development due to increased apoptosis and this phenotype is alleviated but not rescued in a *p53*-deficient background [20,28]. The corresponding heterozygous mice did not show a strong tumor predisposing phenotype [27], even not in a *p53*-deficient background [28]. Due to the aforementioned lethality, conditional models have been extensively used to study *Fancd1/Brca2* tumor suppressor function and have revealed a synergistic effect with *p53* on tumorigenesis [20,35].

Perinatal lethality has been described for several FA mouse models including *Fancc* [36], *Fancd2* [21,37] , *Fancl* [25], *Fancm* [26] , and *Fancp* [32]. This phenotype was strongly dependent on the genetic background of the mice. Especially the C57BL/6J background leads to a more severe perinatal lethality phenotype, as was clearly demonstrated in a recent study where *Fancc* and *Fancd2* deficient mice were underrepresented in this background but not in a mixed 129S4 x C57BL/6J background [37] . For *Fancm* deficient mice an underrepresentation of females was observed, however this effect was also dependent on the genetic background (unpublished results). These data suggest that FA pathway disruptions in mice affect viability when combined with the presence of modifier loci in certain genetic backgrounds (especially in the C57BL/6J background).

Developmental abnormalities

Most FA mouse models did not show any gross FA specific developmental phenotypes with a few exceptions. Microphthalmia has been observed in *Fanca* [16], *Fancc* [36], *Fancd2* [21], and *Fancp* [32] deficient mice in the C57BL/6J background. In addition, a proportion of *Fancp* mice also had an abnormally shaped and enlarged skull [32].

Another notable exception are the gonadal abnormalities and associated infertility, which were observed in all FA mouse models described to date, independent of the genetic background [38,39]. This abnormal function has been ascribed to a reduction in the number of primordial germ cells (PGC) [25,40]. The loss of pluripotent PGCs in FA pathway deficient mice is in agreement with the crucial role for the FA pathway in the derivation or maintenance of induced pluripotent stem (iPS) cells that has been reported recently [41]. It still needs to be determined how this loss of PGCs and poor stem cell self-renewal is related to FA pathway dysfunction. However, it is tempting to speculate that the FA pathway is not only essential for viability after exposure to ICL-

inducing agents, but also for the viability of stem cells. It has been reported that during reprogramming iPS cells are more intolerant to DNA damage and undergo *p53*-mediated apoptosis [42]. *p53* disruption resulted in more efficient reprogramming in the presence of DNA damage [42]. These observations could indicate that the FA pathway might function to maintain genomic stability and prevent DNA damage in pluripotent stem cells. However, in the absence of a functional FA pathway these cells acquire DNA damage and are lost due to apoptosis. Perhaps this loss of pluripotent cells is causative for the growth retardation and short statue observed in humans and FA mice [10,21].

There is also evidence that the FA pathway plays an important role in meiosis. *Fanca* [16] and *Fancd2* [21] deficient mice have an increased incidence of mispaired chromosomes in pachytene spermatocytes accompanied by increased apoptosis. Also the expression of FA proteins is increased during mid-to late-pachytene [16]. During this period chromosomes are fully synapsed, and double Holliday junctions are formed that need to be resolved [43]. The resolution of a double Holliday junction could be aided by the FA pathway, especially by FANCP/SLX4 [44,45]. However, both *Fancp*-deficient male and female mice were able to produce offspring which indicates that meiosis takes place in these mice [32].

Double mutants

To determine whether FA proteins have redundant or divergent functions within the FA pathway, several double FA mutant mice have been generated. Double mutants which phenocopy the single mutant reveal an epistatic relationship, whereas a broader phenotype dictates a non-epistatic relationship. In practice, the interpretation of the phenotype of double FA mutants is less straightforward. The first double mutant mice reported, *Fanca*/*Fancc*, showed no evidence of an additive phenotype, indicating an epistatic relationship between the two genes [17]. However, *Fancc*/*Fancg* double mutant mice did show a broader phenotype as they developed BMF, myelodysplasia and complex cytogenetic abnormalities which were absent in either single knockout mice [46]. This non-epistatic relationship between *Fancc* and *Fancg* was attributed to a unique function for *Fancg* because of the absence of an additive phenotype in the *Fanca*/*Fancc* mice. However, recent data have complicated this interpretation. Mice mutant for both *Fanca* and *Fancg* showed no additive phenotype dictating an epistatic relation between these genes [47]. The different outcomes of epistasis analysis could again be explained by the presence of FA pathway modifiers in different mouse strains. The *Fanca*/*Fancc* and the *Fanca*/*Fancg* were maintained on a 129S4 and mixed 129Ola/FVB genetic background, respectively, whereas the *Fancc*/*Fancg* mice were maintained on a C57BL/6J genetic background. As described above, FA core complex gene defects appear to have a stronger penetrance in mice with a C57BL/6J genetic background [37]. Therefore, C57BL/6J-associated modifier genes may have contributed to the more severe phenotype of the double mutant *Fancc*/*Fancg* mice. The generation of more double mutant mice in different genetic backgrounds, will shed light on the (non)-epistatic relationships between different FA (core complex) genes.

In addition to double mutants with FA core complex genes also mutants with *Fancd2* mice have been generated. A recent study reported a non-epistatic relationship between *Fancd2* and *Fancg*: *Fancd2*/*Fancg* double homozygous mice were not viable in the C57BL/6J background. Also, *Fancd2* but not *Fancg* mutant mice showed an increased frequency of HR mediated deletions at an endogenous locus and more DNA damage and chromosomal instability [48]. This additional role of FANCD2 in genome stability could be related to its role in the intra-S-phase checkpoint. FANCD2 patient cells continue DNA synthesis after IR, indicative of a defective intra-S-phase checkpoint, and this phenotype was not observed in FANCC patient cells and was not dependent on monoubiquitination of FANCD2 [49]. Also FANCD2 is phosphorylated by ATM and CHK1 independent of the FA core complex [49,50]. It is clear that further studies are needed to characterize which phenotypes found in the *Fancd2*-deficient mice extend to other FA core complex mice. The generation and characterization of *Fanci*-deficient mice could also be highly informative to determine to which extent this protein is really FANCD2's sisterly or fraternal twin [51].

Hematological abnormalities

The absence of apparent hematological abnormalities in most of the FA mouse models studied to date is in shrill contrast with the life threatening BMF in FA patients. Exceptions are *Fancp* mice who have lower white blood cell counts and very low levels of platelets and hypomorphic *Fancd1*/*Brca2* mice which had a proliferation defect in the hematopoietic progenitors [32,52]. Bone marrow failure can be induced by treating FA mice with a single high dose of mitomycin C (MMC) or chronic exposure to MMC, as was done with *Fancc* mice [53]. Also degeneration of other highly proliferative tissues such as the liver, spleen, and intestine was observed after a single high MMC dose. This DNA damage induced BMF in FA mice supports the view that mice in laboratory conditions lack natural exposure to stressful environmental agents that contribute to the FA phenotype.

Despite the lack of anemia in most FA mouse models, FA mice have been used to study the role of the FA pathway in hematopoietic homeostasis. This research has focused on hematopoietic stem cells (HSCs), especially in the *Fancc* mouse model. HSCs are multipotent stem cells that can give rise to all blood lineages and can therefore be used in long-term competitive repopulation assays where one compares the ability of FA-HSCs and wild-type HSCs to repopulate the bone marrow of lethality irradiated recipients. This assay did demonstrate a necessity for the FA pathway in hematopoiesis, since *Fancc*-deficient HSCs showed a reduced repopulating ability [54]. *Fancc*-deficient hematopoietic progenitors and stem cells were also sensitive to low doses of inflammatory cytokines such as TNF-α or other inflammatory mediators such as interferon γ and LPS [55-57]. The competitive repopulation assay has also been used as a preclinical tool to study compounds which could enhance its efficiency, as recently demonstrated by improved repopulating ability of *Fancc*-HSCs after treatment with a p38-MAP-kinase inhibitor [58].

Recent data demonstrate that the *Fancd2* mouse model could be especially suitable

to study the contribution of the FA pathway to HSC function and homeostasis. Apart from performing poor in competitive repopulation assays analogous to *Fancc*-deficient HSCs [33,59], *Fancd2*-deficient mice also have a decreased number of HSCs, which was not observed in *Fancc*-deficient mice [59]. Already in 3-week-old *Fancd2* mice, HSCs were underrepresented indicative of an early defect in HSCs development and/or maintenance [59]. The phenotype of *Fancd2*-deficient is more severe than *Fancc*-deficient mice, possibly suggesting that non-ubiquitinated FANCD2 functions in HSC maintenance [59]. *Fancd2*-HSCs also had an increased cell-cycle entry and loss of their normal quiescent state, although this has also been described for *Fancc*-HSCs [60]. It is tempting to speculate that the enhanced cell-cycle entry of HSCs compensates for the lower frequency of HSCs in the bone marrow and thus prevents BMF in the short term. However, in the long-term or after DNA damage (as in MMC treated *Fancc* mice), this accelerated HSC turnover could lead to HSC exhaustion and subsequent BMF. The clear phenotype of *Fancd2*-HSCs makes it a suitable pre-clinical model to study compounds that could reverse this abnormal cell cycle state. The authors already showed that the HSCs phenotype and reduced repopulating ability could be partially rescued by reservatrol, a polyphenol found in grapes and red wine [59].

However, to advise FA patients to indulge in red wine to improve blood counts is likely counter productive advice given recent new data on the involvement of the FA pathway in acetaldehyde toxicity [61]. Aldehydes are reactive metabolites that can induce DNA-DNA and DNA-protein crosslinks and are produced endogenously during normal metabolism or as a byproduct from ethanol metabolism. Aldehyde dehydrogenases (ALDH) are responsible for their detoxification by oxidation of aldehydes to carboxylic acids [62]. In mouse crossing experiments it was shown that in the absence of fetal or maternal ALDH2, *Fancd2* mice were not viable [61]. Double homozygous *Fancd2*/*Aldh2* animals could be obtained from crosses in which at least the mother was *Aldh2*$^{+/-}$, but these mice died within 3 to 6 months due to the development of acute lymphoblastic leukemia (ALL) [61]. In addition, when *Fancd2*/*Aldh2* embryos were exposed to ethanol *in utero* they developed severe physical abnormalities and were underrepresented. Also young *Fancd2*/*Aldh2* animals had a severe reduction in bone marrow cellularity and showed γH2AX induction after exposure to ethanol. These data collectively demonstrate that excess acetaldehydes from endogenous or exogenous sources results in severe toxicity in the absence of FANCD2 [61].

It remains to be determined whether acetaldehyde-mediated toxicity is caused by a specific lesion that needs to be repaired by the FA pathway. In this context it is important to determine whether acetaldehyde toxicity is also seen in other FA mouse models. Mouse embryonic fibroblasts (MEFs) derived from other FA core complex mutant animals were not sensitive to formaldehyde whereas *Fancd2*-deficient MEFs were [63]. However, FA core complex deficient splenocytes or DT40 cells were again sensitive to acetaldehyde [61]. Also, DT40 cells mutant for TLS or HDR were not sensitive to acetaldehyde whereas these proteins are thought to play an important role in repairing acetaldehyde induced crosslinks.

Viability and cancer

The high incidence of anemia, myelodysplasia and cancer reduces the life expectancy of FA patients to 30 years [1,11]. Cancers that frequently arise are acute myeloid leukemia (AML), squamous cell carcinoma (SCC) of the head and neck, SCC of the esophagus, liver tumors, and gynecologic cancers [11-13]. FA mouse models do not recapitulate this pronounced reduction in life expectancy as they generally do not succumb to pronounced accelerated tumorigenesis. For *Fancc*, *Fancd2*, *Fancf*, *Fancm*, and *Fancp* mice, long-term survival cohorts have been published. In the *Fancc*-deficient mice no tumors were observed after 600 days [64]. For the *Fancd2*-deficient mice, a statistically significant increased incidence of both adenomas and carcinomas of epithelial origin was reported [21]. For *Fancf* and *Fancm* mice, a statistically significant reduction in overall and tumor free survival were published [22,26]. *Fancf*-deficient mice developed late onset ovarian tumors, which were potentially related to a deregulation of sex hormones as a consequence of hypogonadism [22]. *Fancm*-deficient mice also showed a reduced life span and increased cancer incidence without skewing to a particular tumor type. *Fancm*-deficient MEFs showed an increased sister chromatid exchange (SCE) frequency, which was not observed in *Fanca*-deficient MEFs and could suggest an additional role for FANCM in genome maintenance [26]. Additional mouse crosses with FA core complex mice are needed to clarify this potential nonepistatic relationship between FANCM and the FA core complex. However, overall, the increased tumorigenesis in the aforementioned mouse models is minor compared to the reduced life expectancy and increased tumor risk in FA patients.

Double mutants between FA mutant mice and non-FA genes

To further uncover genetic interactions between the FA pathway and other cellular pathways, FA mutant mouse models have been crossed to other mouse models. As demonstrated by the *Fancd2*/*Aldh2* mouse study these in vivo genetic interaction studies can shed light on the function of FA genes by enhancing their importance in a sensitized background. Analogous to this approach it was demonstrated that combined *Fancc*/ superoxide dismutase 1(*Sod1*) mutant mice have decreased red blood cell counts and colony forming capacity. SOD metabolizes reactive oxygen species (ROS) which are a source of oxidative damage. Therefore, the aggravated phenotype in these double mutant mice suggest that FANCC functions in counteracting superoxide-mediated genotoxicity or in redox signalling [65].

Genetic interaction studies can also be extended to tumor prone mouse models. By comparing tumor latency, multiplicity, and type between a sensitized background only and the combined mutant one can assess if and how the tumor phenotype is modified. Through these studies it was demonstrated that both *Fancc*- and *Fancd2*-deficiency decreased the latency for tumor development in *p53* mutant mice [64,66]. *p53* heterozygous mice are prone to develop sarcomas whereas homozygous animals mainly develop thymic lymphomas, but also sarcomas and teratomas with a much shorter latency [67]. *Fancc*-deficiency accelerated tumorigenesis in both the *p53* heterozygous and homozygous background [64]. Moreover, *Fancc*/*p53* mice also developed

tumors not observed in *p53* mutants. The combination of *p53*$^{+/-}$ with *Fancd2* loss also resulted in a significant decrease in tumor-free survival, and female animals showed a high incidence of adenocarcinomas [66]. Potentially these tumors were also stimulated by aberrant sex hormone levels as a consequence of hypogonadism as has been suggested for the development of ovarian tumors in *Fancf*-deficient mice [22]. The accelerated tumor formation in *p53*/FA mice suggests that the FA pathway prevents both spontaneous and genotoxic-induced chromosome instability and that activation of *p53* following this DNA damage limits cell proliferation to avoid tumor formation [64,66].

p53/FA mouse models may be suitable to study chemoprevention regimens, which could be of clinical use to FA patients. As an elegant example, tempol was shown to be a potent chemopreventive drug which specifically delayed the onset of epithelial tumors in *Fancd2*-deficient *p53* heterozygous female animals [68].

Concluding remarks

In conclusion, most FA mouse models to date do not fully recapitulate the hallmark characteristics of FA. A notable exception is the *Fancp* mouse model which will undoubtedly be extensively studied in the future. Despite the apparent lack of gross FA symptoms in mice, we have learned a lot of the pathology of FA. The reduction in PGC and consequent hypogonadism as a common phenotype of FA mouse models suggest that the FA pathway is important in early embryogenesis, most likely due to the repair of replication associated stress. Furthermore, the lower number of HSCs in young *Fancd2*-deficient mice suggests that also the development or maintenance of these stem cells is compromised in the absence of an active FA pathway.

Most importantly and encouraging, it appears that FA pathway deficiency in mice does not jeopardize survival under normal circumstances. FA mice seem to thrive in the controlled environment of the laboratory, although the cellular phenotype of ICL sensitivity is maintained in murine FA mutant cells. FA mice most likely thrive because of the absence of harmful environmental exposure or increased detoxification metabolism. Rather, mice seem to display FA phenotypes after excess genotoxic stress from either exogenous agents such as MMC or ethanol or endogenous molecules such as acetaldehyde or ROS. This suggests that FA patients could also benefit from avoiding certain environmental cues. Uncovering the nature of the environmental cues that produce an FA phenotype in mice will undoubtedly stimulate the development of chemopreventive drugs for FA patients in the future.

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Chapter 3

Impaired FANCD2 monoubiquitination and hypersensitivity to camptothecin uniquely characterize Fanconi anemia complementation group M

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Abstract

FANCM is a component of the Fanconi anemia (FA) core complex and one FA patient (EUFA867) with biallelic mutations in *FANCM* has been described. Strikingly, we found that EUFA867 also carries biallelic mutations in *FANCA*. After correcting the FANCA defect in EUFA867 lymphoblasts, a 'clean' FA-M cell line was generated. These cells were hypersensitive to mitomycin C, but unlike cells defective in other core complex members, *FANCM*$^{-/-}$ cells were proficient in monoubiquitinating FANCD2 and were sensitive to the topoisomerase inhibitor camptothecin, a feature shared only with the FA subtype D1 and N. In addition, *FANCM*$^{-/-}$ cells were sensitive to UV light. FANCM and a C-terminal deletion mutant rescued the crosslinker sensitivity of *FANCM*$^{-/-}$ cells, while a FANCM ATPase mutant did not. Since both mutants restored the formation of FANCD2 foci we conclude that FANCM functions in an FA core complex dependent and independent manner.

Introduction

Fanconi anemia (FA) is a recessive genetic instability syndrome that has uncovered a cellular pathway involved in the protection against replication-blocking lesions. Inactivation of this pathway, as seen in FA patients, results in hypersensitivity to DNA cross-linking agents and cancer susceptibility.[1] Defects in 13 different genes have been found in FA patients[2] and the proteins encoded by these genes cooperate in a pathway that can be subdivided in an upstream and downstream part based upon the monoubiquitination of FANCD2 and FANCI.[1] The upstream part of the pathway consists of a nuclear core complex formed by the FA proteins FANCA,-B,-C,-E,-F-G,-L,–M and two FA-associated proteins FAAP100 and FAAP24. This complex monoubiquitinates FANCD2 through the E3-ubiquitin ligase FANCL in conjunction with the ubiquitin conjugating enzyme UBE2T.[3,4] The FA core complex, UBE2T and FANCD2 are independently recruited to the stalled replication fork.[5] For FANCD2 this relies on the ATR-mediated phosphorylation of its binding partner FANCI[6], while the recruitment of the FA core complex seems to depend on FANCM.[7] Like FANCD2, FANCI is also monoubiquitinated by the FA core complex and these modified proteins co-localize with Rad51 and BRCA1 in nuclear foci.[8,9] The link between FA and BRCA proteins was further strengthened by the discovery of FA patients with mutations in BRCA2[10], and in the BRCA1- and BRCA2-interacting proteins BRIP1[11,12] and PALB2[13,14]. FA patients with a defect in any of these genes have normal FANCD2 monoubiquitination and therefore these proteins are considered as downstream players in the FA pathway.

Despite the identification of the various components of the FA core complex its role in the maintenance of genome stability remains unclear, because of the absence of functional domains in most of the core complex members. A notable exception is FANCM, an ortholog of the archaeal DNA repair protein HEF, which contains two conserved domains: a DEAH helicase domain in the N-terminus and an endonuclease domain in the C-terminus.[15,16] The helicase domain is shared with yeast orthologs MPH1 (*S. cerevisiae*) and FML1 (*S. pombe*), which play a regulatory role in homologous recombination repair by replication fork reversal and D-loop disruption.[17-19] HEF and

MPH1 possess helicase activity[20,21], while for FANCM only translocase activity was observed.[15] Purified FANCM also promotes branch migration of Holiday junctions and replication forks[22] and remodels DNA replication structures[23], establishing a possible link between the FA pathway and DNA repair.

FANCM, initially known as FAAP250, was identified as a FANCA-interacting protein in an attempt to purify the FA core complex.[24] FAAP250 was named FANCM after an unclassified FA patient (EUFA867) with biallelic mutations in the FAAP250-encoding gene was found.[15] Lymphoblasts from this patient lacked FANCM protein expression and FANCD2 monoubiquitination. In addition, FANCA and FANCG levels were reduced and the nuclear localization of FANCA and FANCL was disturbed. EUFA867 had been excluded from other known complementation groups by linkage analysis, cell fusion and cDNA transfection and was therefore assigned to a new complementation group FA-M. Curiously, experiments designed to complementation EUFA867 lymphoblasts with FANCM cDNA to reverse crosslinker sensitivity, have reproducibly failed.

We now report that EUFA867, in addition to biallelic *FANCM* mutations, also has biallelic mutations in *FANCA*. Correction of the FANCA defect revealed the features specific for FA-M cells i.e. hypersensitivity to DNA crosslinking agents and to the topoisomerase I inhibitor camptothecin, which could be rescued by ectopic expression of FANCM. At the molecular level, FANCM deficient lymphoblasts show residual FANCD2 monoubiquitination, indicating that FANCM is required both for efficient FANCD2 monoubiquitination and in the DNA repairs, later in the FA pathway.

Methods

Cell lines and culture conditions

Epstein-Barr virus-transformed lymphoblasts were cultured in RPMI1640 medium or Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum. EUFA867 lymphoblasts stably expressing FANCA were generated by electroporation of an episomal pMEP4 construct encoding FANCA with a C-terminal flag-tag and selection on hygromycin, by transduction with retroviral vector pMMP-FANCA and selection on puromycin, or by transduction with bicistronic retrovirus SF91-FANCA and sorting transduced EGFP positive by FACS. The MMC-, camptothecin- and UV-induced growth inhibition assays as well as the cell fusions were performed as previously described.[25]

Retroviral infections

The pMIEG3 bicistronic retroviral vector, bicistronic retroviral vectors expressing FA proteins and pMMP-FANCA have been described.[26,27] FANCM was cloned into BamHI and XhoI sites of pMIEG3 to generate pMIEG3-FANCM. Similarly K117R-FANCM and delC-FANCM were subcloned into pMIEG3. Retroviral vectors SF91-FANCM (without IRES-EGFP) and SF91-FANCA (with IRES-EGFP) were also constructed.

To prepare transient amphotropic virus stocks, 293T cells (1.5×10^6) were plated in 10 cm dishes. The next day, cells were transfected with the retroviral expression vectors and the appropriate helper plasmid (gag-pol and RD114) using lipofectamine 2000. The medium was changed 5 hours after transfection and retrovirus-containing medium was harvested in 12 hour increments at 24 hours post-transfection.

Lymphoblasts were transduced in six-well plates coated with fibronectin fragment CH-296 (8 Ag/cm2; Takara Shuzo, Otsu, Japan). Approximately 5x105 cells per well were exposed twice to the virus supernatant for 6 h26. Subsequently, the transduced cells were removed from the wells using cell dissociation buffer (Invitrogen, Grand Island, NY, USA) and transferred to new plates containing fresh medium. Two days after transduction, the transduced cells were sorted based on the EGFP expression using a FACS Vantage cell sorter (BD Biosciences, San Jose, CA, USA).

Antibodies

Anti-FANCM, -FANCA, -FANCL and -FANCD2 were described.[15,28] Anti-FANCG and anti-FAAP24 were kind gifts from Fanconi Anemia Research Fund and Dr. Steve West, respectively. Anti-histone H2A antibody was purchased from Millipore (Billerica, MA, USA).

FANCD2 immunofluorescence

Cells were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature (RT) and then deposited on 12 mm diameter glass coverslips coated with poly-D-lysine by centrifugation in a Thermo Shandon Cytospin apparatus (1200 rpm, 2 min). After washing with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 3 min, and washed again with PBS. Then anti-FANCD2 antibody E35 (1:200) in antibody buffer (PBS/3% BSA/0.05% Tween 20/0.04% sodium azide) was added for 1 hr at 37°C with. Cells were washed with PBS and incubated for 30 min at 37°C with Rhodamine B-conjugated donkey anti-rabbit IgG antibody (Jackson Immunoresearch; 1:200). Cells were washed with PBS, mounted by Vectashield with 4',6-diamino-2-phenylindole (DAPI) to stain DNA and analyzed with a Zeiss Axiovert 200M microscope.

RAD51 immunofluorescence

Lymphoblasts were transferred to 9-cm culture dishes at a density of $0.5\text{x}10^6$ cells/ml and either mock-treated or treated with MMC (2.4 µg/ml for 1 h) or X-ray irradiation (12 Gy). Cells ($1\text{x}10^6$) were seeded on poly-D-lysine-coated glass slides 7 or 24 h after recovery from the treatment and left to attach for 15 minutes prior to fixation with 2% formaldehyde in PBS. Then cells were permeabilized with PBS/0.1% Triton X-100. Subsequently, the slides were blocked for 30 min in PBS/0.5% BSA/0.15% glycin and incubated with anti-Rad51 antiserum FBE2 for 90 min. The slides were washed in PBS/0.1% Triton X-100 and incubated with AlexaTM 488-conjugated goat anti-rabbit (Molecular Probes) for 1 h at 37°C. After 3 washes with PBS/0.1% Triton X-100 the cells were counterstained with DAPI in Vectashield mounting medium and analyzed.

Preparation of cellular fractions

Cellular fractionation was as described.[7] The cells were trypsinized and washed with ice cold PBS. Pellets were resuspended in cold buffer A (10 mM PIPES (pH 7.0), 100 mM NaCl, 300 mM sucrose, 1 mM EGTA, 0.5% Triton X-100, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, protease inhibitor cocktail, 1 mM PMSF) at five times the volume of the cell pellet and incubated for 2 min at RT to permeabilize the cells. The suspension was centrifuged at 200g for 3 min, and the supernatant S100 (detergent-soluble cyto-nucleoplasmic proteins) was collected. Pellets were washed with cold buffer A and extracted with buffer B (buffer A with 400mM NaCl) for 5 min to obtain supernatant S400 (chromatin fraction).

Cell cycle analysis

To induce cell cycle arrest, approximately 1x106 cells were plated in culture medium containing 0.5 µg/ml of the cross-linking agent melphalan (Sigma, St. Louis, MO, USA) for 48 h. Cells were fixed for 20 min at RT in PBS/1% paraformaldehyde. Subsequently, cells were washed with PBS and resuspended for 10 min at RT in PBS/0.1% Triton X-100. After washing with PBS, cells were stained for 1 h at 4oC with a solution of PBS containing 2 mg/ml RNase A (Qiagen, Valencia, CA, USA) and 50 mg/ml propidium iodide (Sigma). Flow cytometry was performed on a FACS Calibur flow cytometer (BD Biosciences) and analysis of the percentage of cells in each phase of the cell cycle was performed using the MODFIT-LT software.

Drug sensitivity assay

Lymphoblasts (10^4) were cultured continuously for 5 days in 100 µl medium containing different concentrations of drug. Cell viability was measured using the chromogenic Cell Titre 96 Proliferation Assay (Promega Corporation, Madison, WI) recording the optical density at 490 nm.

Results

FANCA mutations in patient EUFA867

The reference FA-M patient EUFA867 carries two pathogenic mutations in *FANCM*. As expected for cells with a defect in core complex proteins in the FA pathway, lymphoblasts from this patient, which do not express FANCM protein, lack monoubiquitinated FANCD2.[15] Surprisingly, ectopic FANCM expression was unable to restore FANCD2 monoubiquitination (Figure 3.1A, lane 1 and Supplementary Figure S3.1A), suggesting an additional defect in this cell line. Expression of FANCA, but not of other FA proteins, in FANCM-expressing EUFA867 cells completely rescued FANCD2 monoubiquitination (Figure 3.1A, lane 2), pointing to a FANCA defect in these lymphoblasts. This idea was strengthened by cell fusion experiments between EUFA867 lymphoblasts and FANCA deficient HSC72 lymphoblasts, resulting in tetraploid cell hydrids that remained MMC-sensitive (Supplementary Figure S3.1B), whereas fu-

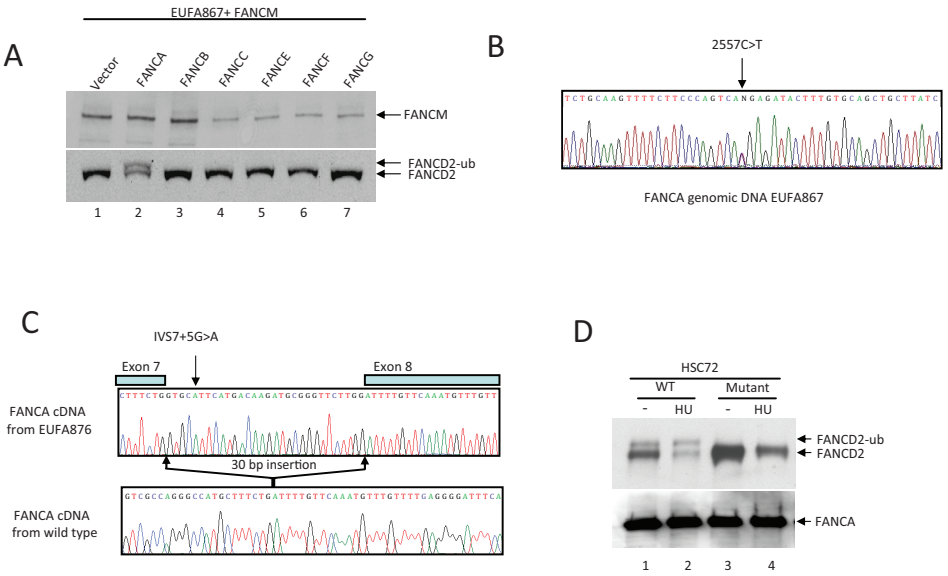

FIGURE 3.1

EUFA867 has biallelic FANCA mutations. (A) Introduction of FANCA in EUFA867 lymphoblasts stably expressing FANCM restores FANCD2 monoubiquitination. EUFA867 lymphoblasts were transduced with SF91-FANCM to obtain EUFA867 + FANCM. Subsequently these cells were transduced with bicistronic retroviruses encoding the indicated FA proteins. Cells were treated with 2 mM HU for 16 h and immunoblotted for FANCD2 and FANCM. (B) EUFA867 has a nonsense mutation 2557C>T (R853X) in FANCA. (C) Mutation IVS7+5G>A affects the normal splice donor in FANCA intron 7 and results in a 30 bp insertion of intron 7 sequence in the cDNAs of EUFA867 and her mother. The sequence shown is from an isolated cDNA clone of EUFA867. (D) FANCA cDNA derived from the IVS7+5G>A mutation does not correct FANCD2 monoubiquitination. FANCA deficient HSC72 lymphoblasts were transduced with bicistronic retrovirus encoding either wild type FANCA or the FANCA mutant derived from the mutation IVS7+5G>A. Cells were treated with 1 mM HU to stimulate FANCD2 monoubiquitination.

sion hybrids with the FANCC-deficient cell line HSC536 were MMC-resistant (Supplementary Figure S3.1C). Indeed, sequencing revealed biallelic *FANCA* mutations in the EUFA867 cell line: a nonsense mutation (2557C>T, R853X), inherited from the father (Figure 3.1B and Supplementary Figure S3.2), and a splice-site mutation in intron 7 (IVS7+5 G>A), inherited from the mother (Figure 3.1C and Supplementary Figure S2). These mutations were confirmed in blood DNA of the patient and the parents, as well as in DNA from lymphoblastoid cell lines of the parents. The splice-site mutation, which has been observed previously[29,30], leads to a 30 bp insertion in the FANCA mRNA (Figure 3.1C). The insertion does not affect the FANCA open reading-frame and adds 10 amino acids between amino acids 236 and 237 of the FANCA protein. However, the mutant FANCA protein was unable to restore FANCD2 monoubiquitination in FANCA deficient lymphoblasts (Figure 3.1D), demonstrating that the splice-site mutation is pathogenic. The insertion is near the amino acids L210 and L274, which when mutated result in loss of function by abolishing the nuclear accumulation of FANCA.[28] Since subcellular fractionation experiments in EUFA867

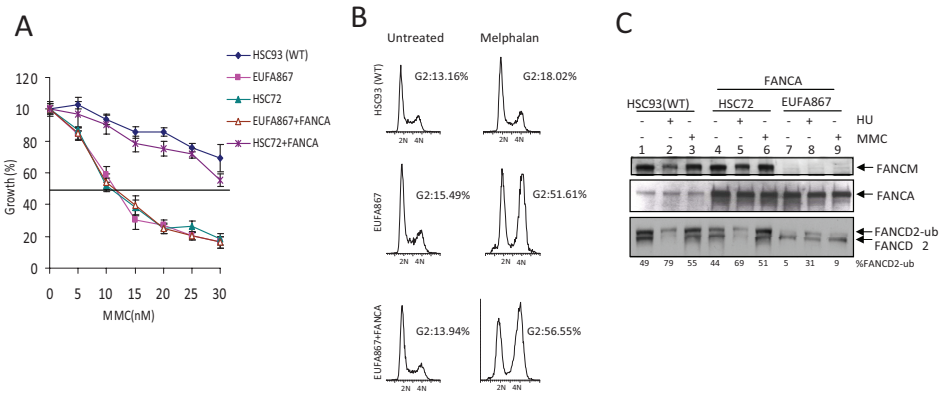

FIGURE 3.2

Stable expression of FANCA in EUFA867 lymphoblasts partially corrects the phenotype of this cell line. (A) EUFA867 lymphoblasts stably expressing wild type FANCA are still hypersensitive to growth inhibition by mitomycin C. FANCA-deficient HSC72 and EUFA867 lymphoblasts were transduced with SF91-FANCA. Viable cells were measured with the Cell Titre 96 Proliferation Assay. The data represent the percentage growth compared to untreated cells and show one representative result of three independent experiments with standard deviations. (B) EUFA867 lymphoblasts stably expressing wild type FANCA show melphalan-induced G2 arrest. (C) EUFA867 lymphoblasts stably expressing wild type FANCA have reduced FANCD2 monoubiquitination. Cells were treated with either 2 mM HU or 240 nM MMC for 16 hr or left untreated. Total lysates were immunoblotted for FANCM, FANCD2 and FANCA.

cells failed to detect FANCA in the nuclear fraction[15], the insertion probably affects the nuclear localization of FANCA and the interaction with other FA core complex members.

FANCM deficiency leads to crosslinker sensitivity and reduced FANCD2 monoubiquitination

These new genetic data led us to re-examine the role of FANCM in the FA pathway. We first corrected the FANCA defect in EUFA867 lymphoblasts to create a cell line that is only deficient in FANCM. FANCA expression by either retroviral transduction or cDNA transfection did not restore MMC hypersensitivity in EUFA867 cells (Figure 3.2A and Supplementary Figure S3A). In addition, the G2 arrest observed in EUFA867 cells after melphalan treatment was still present in the FANCA-expressing cells (Figure 3.2B), suggesting that FANCM deficiency is associated with crosslinker hypersensitivity. FANCA expression partially rescued the FANCD2 monoubiquitination defect seen in EUFA867 cells (Figure 3.2C and Supplementary Figure S3B), which is very similar to the situation observed in FANCM-deficient mouse embryonic fibroblasts (Chapter 4, this thesis). Strikingly MMC induced monoubiquitination of FANCD2 is more severely impaired in FANCA expressing EUFA867 cells than HU induced monoubiquitination. (Figure 3.2C, lanes 8,9 and Figure 3B, lanes 1,2). These data indicate that FANCM is not essential for FANCD2 monoubiquitination, but may increase the efficiency of this process.

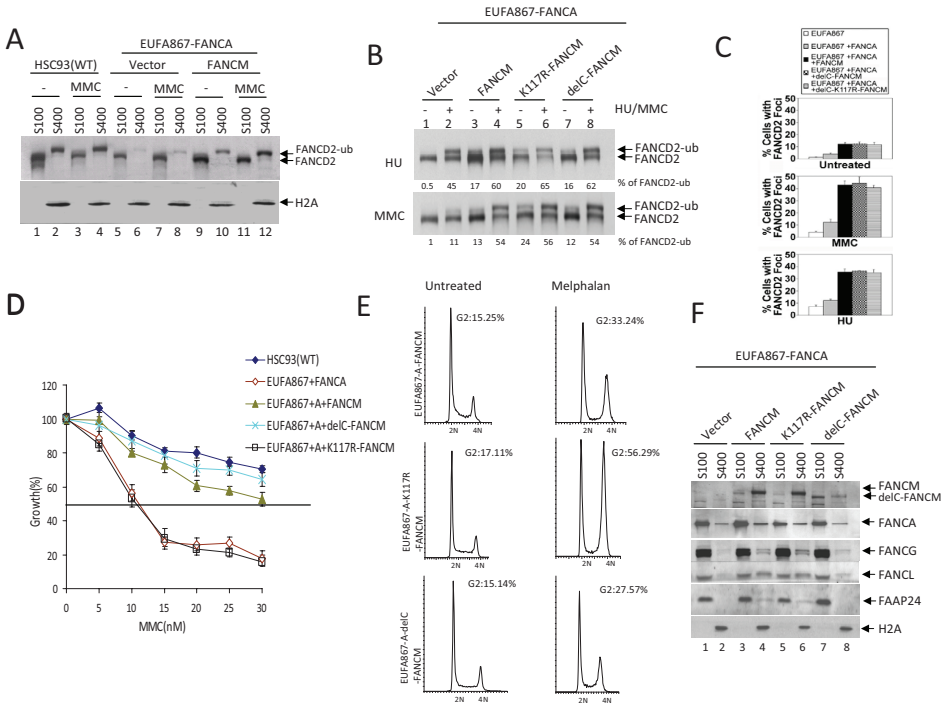

FIGURE 3.3

Ectopic expression of both FANCA and FANCM can correct the FA phenotype of EUFA867 lymphoblasts. (A) Ectopic expression of FANCA and FANCM in EUFA867 restores the chromatin localization of monoubiquitinated FANCD2, which coincides with an enhanced FANCD2 monoubiquitination. EUFA867 cells stably expressing FANCA (EUFA867-FANCA) were generated with pMMP-FANCA. Subsequently, these cells were transduced with either MIEG3 bicistronic retroviral vector or MIEG3 encoding wild type FANCM. EGFP-positive cells were treated with 240 nM MMC for 16 hr or left untreated and subcellular fractions were made; S100 cytoplasmic and nucleoplasmic proteins, S400 chromatin-bound proteins. (B) The ATPase activity and the C-terminus of FANCM are not required for efficient FANCD2 monoubiquitination. EUFA867 lymphoblasts stably expressing FANCA were transduced with either MIEG3 retroviral vector or MIEG3 encoding wild type FANCM, an ATPase-dead FANCM mutant (K117R-FANCM) or a C-terminal FANCM deletion mutant (delC-FANCM). EGFP-positive cells were treated with either 2 mM HU (upper panel) or 240 mM MMC (lower panel) for 16hr. Total lysates were immunoblotted for FANCD2. (C) FANCM, but not its ATPase activity or its C-terminus is required for the assembly of FANCD2 foci. Cells were either left untreated or exposed to 450 nM MMC or 2 mM HU for 24 hr and the percentage of cells with 5 or more FANCD2 foci was determined in at least 150 cells. The result shows the average of three independent experiments with standard deviations. (D) The ATPase activity, but not the C-terminus of FANCM is required for MMC resistance. Viable cells were measured with the Cell Titre 96 Proliferation Assay. The data represent the percentage growth compared to untreated cells and show one representative result of three independent experiments with standard deviations. (E) The FANCM ATPase mutant does not rescue the melphalan-induced G2 arrest in EUFA867 lymphoblasts. (F) FANCM is required for chromatin targeting of the FA core complex proteins. Subcellular fractions of EUFA867 lymphoblast and stably transduced derivatives were immunoblotted for FANCM, FANCA, FANCG, FANCL and FAAP24. H2A was used as a loading control for the chromatin fraction.

To further investigate the function of FANCM, chromatin fractions of FANCA-transfected EUFA867 cells were analyzed and compared to EUFA867 cells in which both FANCA and FANCM defects were corrected. In the absence of FANCM less monoubiquitinated FANCD2 was found in the chromatin fraction, which could be restored by FANCM expression (Figure 3.3A). This increased chromatin binding coincided with an enhanced FANCD2 monoubiquitination (Figure 3B), the appearance of FANCD2 foci (Figure 3.3C), a restoration of the MMC hypersensitivity (Figure 3.3D) and a reduction of melphalan induced G2 arrest (Figure 3.3E). In addition, FANCG, FANCL, and FAAP24 levels, which were decreased in the chromatin fraction of FANCA-corrected EUFA867 cells, were normalized by FANCM expression (Figure 3.3F). As has been suggested from siRNA experiments in HeLa cells[7] our data show that FANCM assists the recruitment of the FA core complex to the chromatin, which then may increase FANCD2 in chromatin bound foci through monoubiquitination.

The ATPase activity of FANCM is essential for crosslinker tolerance.

FANCM contains two interesting motifs: a N-terminal DEAH helicase domain and a C-terminal ERCC4-like nuclease domain. The involvement of these domains in crosslinker resistance was investigated by expressing either a FANCM ATPase point mutant (K117R) or a C-terminal FANCM deletion mutant in FANCA-corrected EUFA867 cells. Like wild type FANCM, both mutants restored the monoubiquitination of FANCD2 and its ability to form nuclear foci (Figure. 3.3B, 3.3C). However, only the C-terminal deletion mutant was able to restore MMC resistance (Figure 3.3D) and melphalan induced G2 arrest (Figure 3.3E). These data confirm previous siRNA experiments in HeLa cells, showing that the K117R mutant supports normal FANCD2 and FANCI monoubiquitination, but is unable to induce crosslinker resistance.[31] Interestingly, this ATPase point mutant assisted in the recruitment of FANCG, FANCL and FAAP24 to the chromatin (Figure 3.3F), indicating that the ATPase activity of FANCM is essential for crosslinker resistance by supporting a step that is independent of the FA core complex..

The C-terminal deletion mutant seems to have a reduced chromatin association and only partially supports the chromatin binding of FANCG and FANCL (Figure 3.3F), but apparently this is sufficient to complement crosslinker sensitivity. In line with the role of the FANCM C-terminus in binding FAAP24[32], FAAP24 is absent in the chromatin fraction of cells transfected with the C-terminal deletion mutant (Figure 3.3F). This could imply that, in contrast to HeLa cells, in which knockdown of FAAP24 by siRNA resulted in a crosslinker-sensitive phenotype[32], lymphoblasts may not depend on FAAP24 for crosslinker resistance. The same could hold for the chicken B cell line DT40, since ablation of the FANCM C-terminus in these cells resulted in a moderate level of crosslinker sensitivity.[16]

FANCM deficiency is associated with camptothecin and UV sensitivity

Recently it has been shown that purified FANCM can promote branch migration of Holliday junctions and replication fork.[22] Therefore, it was postulated that FANCM,

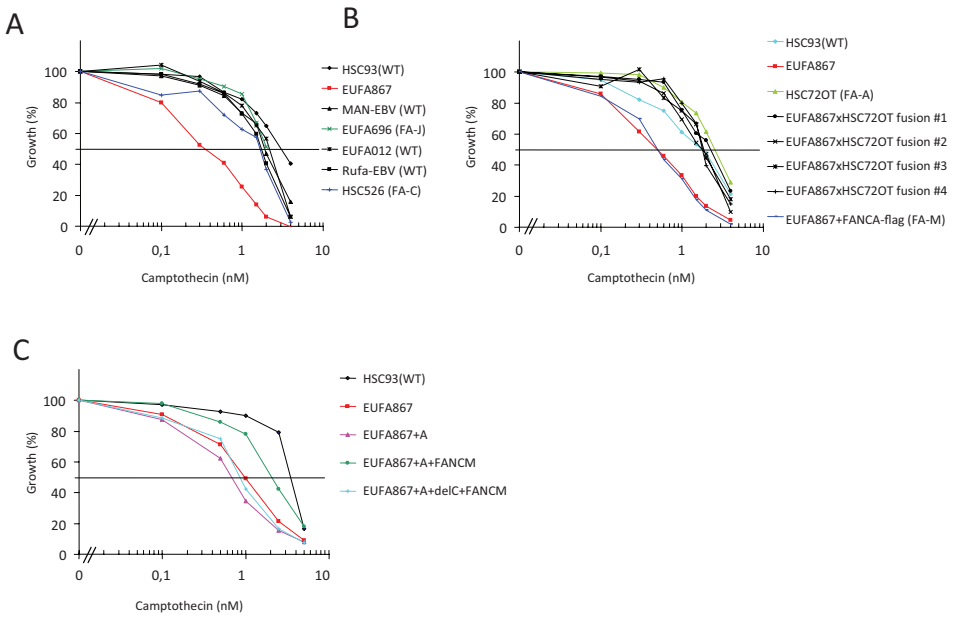

FIGURE 3.4

Camptothecin sensitivity in human lymphoblasts. (A) EUFA867 lymphoblasts are sensitive to the topoisomerase I inhibitor camptothecin. Lymphoblast were continuously exposed to different doses camptothecin and cell growth was compared to untreated cells by cell counting. Wild type, FANCC- and FANCJ-deficient lymphoblasts were included as camptothecin resistant controls. (B) Camptothecin sensitivity of EUFA867 lymphoblasts is due to a defect in FANCM. The FANCA defect in EUFA867 was corrected by stable transfection of flag-tagged FANCA (EUFA867+FANCA-flag), the FANCM defect was corrected by cell fusion with FANCA deficient lymphoblasts HSC72 (EUFA867xHSC72OT fusion). Four independent cell fusions are depicted. (C) The C-terminus of FANCM is involved in camptothecin resistance. EUFA867 cells were stably transduced with FANCA and wild type FANCM (FANCM) or a C-terminal deletion mutant of FANCM (delC-FANCM).

apart from its function in the FA pathway, might have additional functions in the remodelling of replication intermediates. We speculated that if FANCM indeed has additional functions, cells deficient for FANCM might be sensitive to a broader range of genotoxic agents. Therefore, we tested various DNA damaging agents in growth inhibition assays on EUFA867 lymphoblasts and found that this cell line was hypersensitive to the topoisomerase I inhibitor camptothecin (Figure 3.4A). This hypersensitivity was specific for FANCM deficiency, since FANCA overexpression in EUFA867 cells did not restore camptothecin resistance, whereas introduction of FANCM by fusion of EUFA867 lymphoblasts with FANCA- or FANCC-deficient lymphoblasts corrected this phenotype (Figure 3.4B and Supplementary Figure S3.4C). As expected from these cell fusion data, wild type FANCM restored the camptothecin resistance, but surprisingly the C-terminal deletion mutant did not correct this defect (Figure 3.4C). This indicates that the C-terminus of FANCM is involved in the recovery of replication forks stalled by topoisomerase I-DNA cleavage intermediates fixed on the DNA by topoisomerase I inhibitors.

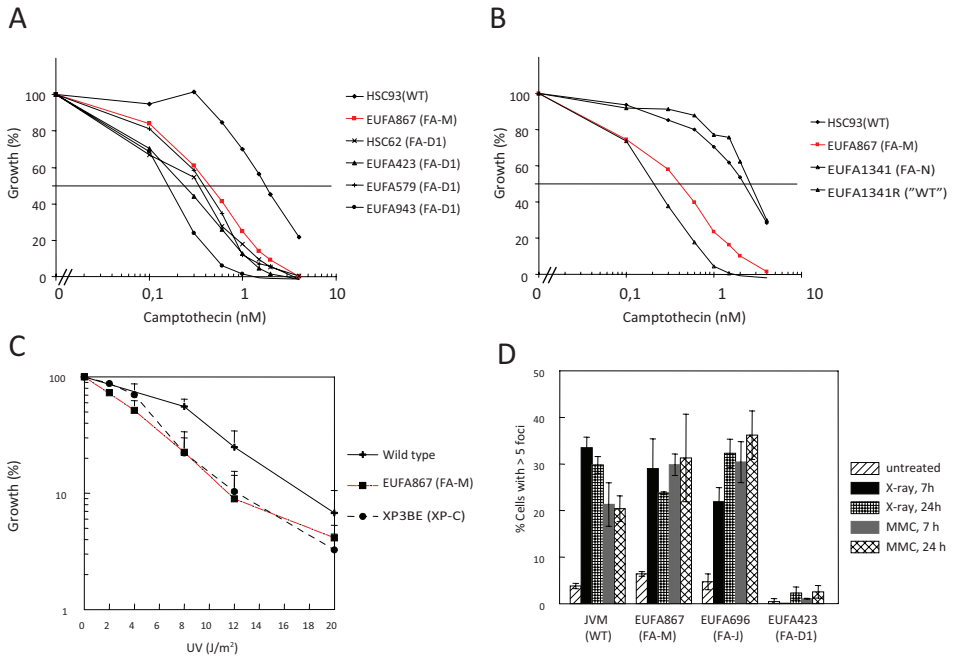

FIGURE 3.5

Camptothecin and UV sensitivity and Rad51 focus formation in human lymphoblasts. (A) FA-D1 lymphoblasts with a defect in FANCD1/BRCA2 are as sensitive to growth inhibition by camptothecin as EUFA867 lymphoblasts. (B) FA-N lymphoblasts with a defect in FANCN/PALB2 are as sensitive to growth inhibition by camptothecin as EUFA867 lymphoblasts. EUFA1341R is a MMC resistant reverted derivative of EUFA1341 lymphoblasts. (C) EUFA867 lymphoblasts are sensitive to UV. Lymphoblasts were exposed to different doses UVC light and cell growth was compared to untreated cells by cell counting. Lymphoblasts of xeroderma pigmentosum patient XP3BE were used as a positive control. Results show mean values of at least 5 experiments with standard error of the mean. (D) Normal Rad51 focus formation in EUFA867 lymphoblasts. Kinetics of Rad51 foci formation in response to X-ray irradiation (12 Gy) or MMC treatment (2.4 µg/ml for 1h) analyzed 7 and 24 h after treatment. A cell with more than five distinct foci in the nucleus was considered positive. Results show the mean values of at least 2 experiments with standard error of the mean.

The camptothecin sensitivity is specific for EUFA867 lymphoblasts and was not found in lymphoblasts with a defect in other FA core complex members or in FANCD2, FANCI or FANCJ (Figure 3.4A, Supplementary Figure S3.4D and S3.4G). In contrast, we observed camptothecin sensitivity in 4 independent FANCD1/BRCA2 deficient lymphoblasts (Figure 3.5A) and in FANCN/PALB2 deficient lymphoblasts (Figure 3.5B). These results suggest that FANCJ and BRCA2/PALB2 function in two different branches of the FA pathway and that FANCM might have a role in the BRCA2/PALB2 branch, which is thought to be connected to homologous recombination. Like BRCA2-deficient cells[33], EUFA867 cells were found to be UV-sensitive (Figure 3.5C), which is probably not due to the FANCA defect in this cell line since FANCA deficient cells were shown to be UV resistant33. However, the normal formation of DNA damage-induced Rad51 foci in EUFA867 cells (Figure 3.5D) indicates that, unlike BRCA2 and PALB2, FANCM does not play an essential role in homologous recombination repair.

Discussion

FANCM was classified as an FA gene based upon the discovery of pathogenic mutations in FA patient EUFA867, but ectopic FANCM expression in lymphoblasts from the patient has been unable to rescue its cellular phenotype.[15] Here we show that this was probably due to an additional FANCA defect in this patient. Stable FANCA expression in EUFA867 cells revealed the consequences of FANCM deficiency, e.g. hypersensitivity to DNA crosslinking agents and to the topoisomerase I inhibitor camptothecin as well as impaired FANCD2 monoubiquitination. In addition, correction of the FANCA defect now allowed functional complementation studies with FANCM and FANCM mutants, which showed that the C-terminus of FANCM is essential for camptothecin resistance, but dispensable for crosslinker tolerance.

Our data indicate that patient EUFA867 is a very exceptional case, with two pathogenic *FANCA* mutations besides the biallelic *FANCM* mutations. Remarkably, the sibling of EUFA867, who was the first to be diagnosed with FA following the development of typical FA features, carried the same biallelic mutations in *FANCA*, but carried only one of the *FANCM* mutations, which in retrospect classifies this patient as an FA-A patient (Supplementary Figure S3.2). EUFA867 was not suspected to have FA, since she failed to display any conspicuous clinical symptoms typical for FA. The basis of her diagnosis was the result of a chromosomal breakage assay performed because of the diagnosis of her brother. Although in general clinical features of FA patients are highly variable, the atypical appearance of EUFA867 may suggest that patients with a FANCM defect present with a different phenotype. As FANCM appears to be essential for the core complex functions[7], the FANCM deficiency in EUFA867 may have overruled the FANCA defect and changed the clinical outcome in this patient. A similar situation has been observed in DT40 cells, in which disruption of FANCM in a FANCC-deficient background attenuates cisplatin toxicity.[16] This hypothesis is strengthened by the atypical phenotype of FANCM deficient mice (Chapter 4, this thesis) and may explain why the number of FA patients diagnosed with a FANCM defect is still limited to patient EUFA867. Our data warrant FANCM mutation screening in related syndromes of which the disease gene has not been identified.

Although the absence of FANCM may lead to a phenotype different from defects in other FA core complex members, FANCM deficient cells showed crosslinker sensitivity and lacked FANCD2 foci, indicating that FANCM plays an essential role in the FA pathway. The residual monoubiquitination observed in EUFA867 lymphoblasts stably expressing FANCA suggests that this role does not involve the formation of the FA core complex. Chromatin fractionation studies in the FANCA-corrected EUFA867 cells indicated that FANCM is rather responsible for the recruitment of the FA core complex to the chromatin, which may than allow efficient FANCD2 monoubiquitination and focus formation. This function does not require ATP hydrolysis, since the K117R FANCM mutant was able to support this step and also the C-terminus of FANCM appeared to be dispensable for this process. The FANCM mutants do point to a role for FANCM in the DNA repair in the later part of the FA pathway after FANCD2 monoubiquitination. The K117R mutant did not restore MMC resistance,

indicating that an ATP dependent step is needed after FANCD2 is monoubiquitinated, which probably involves the ATPase dependent remodelling of the stalled replication fork.[23] The C-terminus of FANCM was not critical for crosslinker resistance, but seemed to be essential for the repair of camptothecin induced DNA damage, possibly through its interaction with FAAP24.

The camptothecin sensitivity of FANCM deficient lymphoblasts was shared with lymphoblasts defective in BRCA2 or PALB2, thus suggesting a role for FANCM in the DNA repair branch of the FA pathway connected to homologous recombination repair. However, unlike lymphoblasts with a mutation in BRCA2 or PALB2, EUFA867 lymphoblasts showed normal Rad51 foci, indicating that FANCM is not essential for homologous recombination repair. FANCM may have a more regulatory function in the homologous recombination process as suggested by the role of the yeast FANCM orthologs MPH1 (*S. cerevisiae*) and FML1 (*S. pombe*), in replication fork reversal and D-loop disruption.[17-19] The increased sister chromatid exchanges in FANCM deficient DT40 cells[16] and FANCM deficient mouse embryonic fibroblasts (Chapter 4, this thesis) hints at a role for FANCM in the suppression of crossover recombination.

In summary, we have shown that EUFA867 lymphoblasts are defective in two distinct FA core complex components, FANCA and FANCM, which explains why FANCM expression has been ineffective in complementing this cell line. This discovery also clarifies the apparent discrepancies with regard to FA core complex stability and FANCD2 monoubiquitination between EUFA867 lymphoblasts and HeLa cells depleted of FANCM by siRNA[7,31] as well as FANCM DT40 knock-outs[16]. Correction of the FANCA defect in EUFA867 cells partially restored FANCD2 monoubiquitination, but these cells are still crosslinker sensitive. EUFA867 lymphoblasts were also hypersensitive to camptothecin and UV light, a phenotype not previously known to be associated with FANCM deficiency. FANCD2 monoubiquitination and focus formation as well as crosslinker and camptothecin sensitivity were restored by ectopic FANCM expression. However, a C-terminal FANCM deletion mutant only normalized FANCD2 monoubiquitination and crosslinker sensitivity, but not the camptothecin sensitivity. The ATPase activity of FANCM is required for crosslinker resistance but not for FANCD2 monoubiquitination and focus formation. Our data thus suggest specific roles for different parts of the FANCM protein in the DNA damage response, both for efficient FANCD2 monoubiquitination and DNA repair steps later in the FA pathway.

Acknowledgements

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Authorship contribution

TRS designed and performed research, STB performed research and edited the paper, SA performed research and edited the paper, MJ performed research, EG performed research, BCG performed research and edited the paper, AMA performed research, CD performed research, MAR performed research, QF performed research, KW performed research, JS performed research, PRA performed research, DAW designed research, controlled data, HJ controlled data and edited the paper, JPW designed research, controlled data and wrote the paper, ARM designed research, controlled data and wrote the paper.

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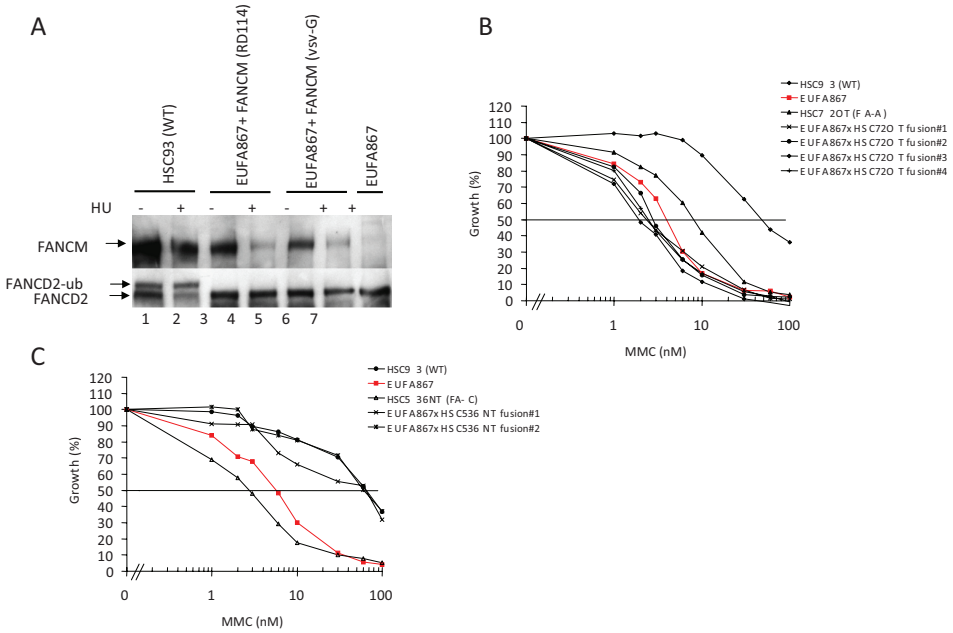
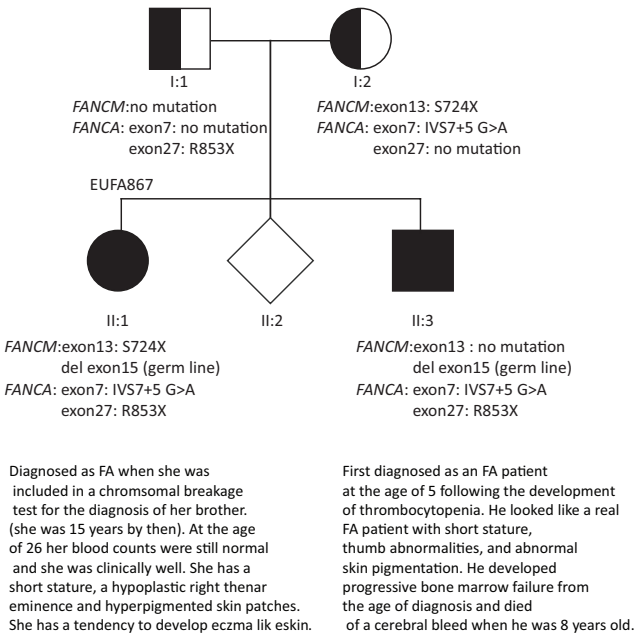
Supplementary Data



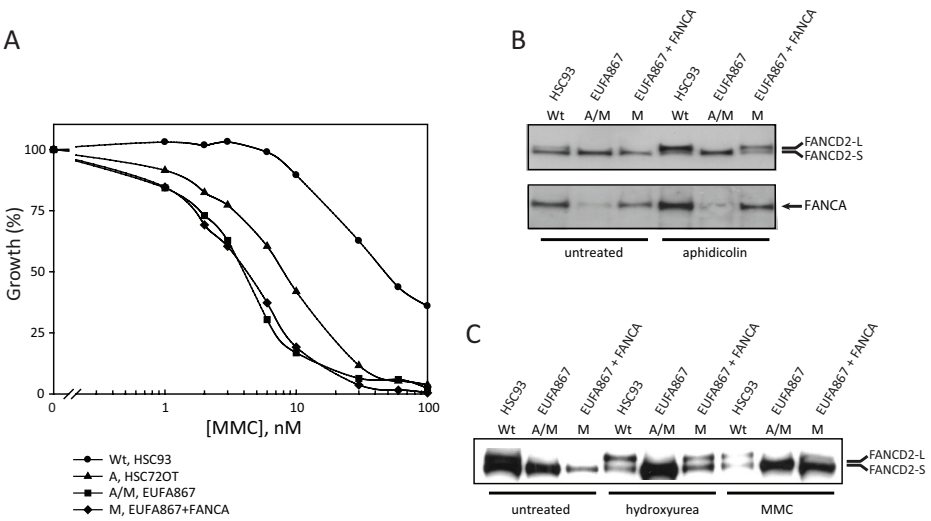
Supplementary Figure S3.1

(A) Ectopic expression of FANCM is not sufficient to restore FANCD2 monoubiquitination in EUFA867 cells. Lymphoblasts were transduced with retroviruses of either VSG or RD114 pseudotypes expressing FLAG-tagged FANCM. The transduced cells were either treated with 1mM HU for 16 h or left untreated and the total lysates were immunoblotted for FANCD2 and FANCM proteins. HSC93 (wild type) and EUFA867 lymphoblasts were used as positive and negative controls respectively. B) Cell fusion between EUFA867 and FANCA deficient HSC72 lymphoblasts does not correct the MMC hypersensitive phenotype of EUFA867 cells. Lymphoblast were continuously exposed to different doses MMC and cell growth was compared to untreated cells by cell counting. Four independent cell fusions are depicted. HSC72 is the reference cell line for FA complementation group A and is able to complement the MMC hypersensitive phenotype of all FA cell lines, except those that have a defect in FANCA. HSC93 is included as wild type control lymphoblasts. (C) Cell fusion between EUFA867 and FANCC deficient HSC536 lymphoblasts does correct the MMC hypersensitive phenotype of EUFA867 cells. Two independent cell fusions are depicted. HSC536 is the reference cell line for FA complementation group C and is able to complement the MMC hypersensitive phenotype of all FA cell lines, expect those that have a defect in FANCC. HSC93 is included as wild type control lymphoblasts.



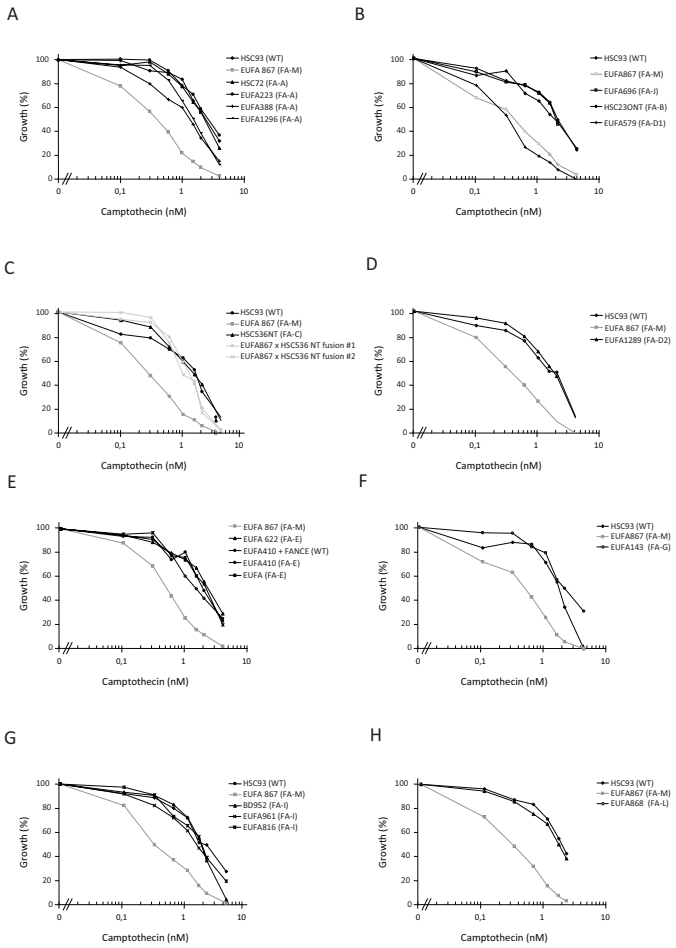
Supplementary Figure S3.2

Pedigree of the EUFA867 family and overview of mutations and clinical phenotype. Fanconi anemia patient EUFA867 (subject II:1) has two pathogenic mutations in *FANCM* and two pathogenic mutations in *FANCA*. The nonsense mutation S724X in exon 13 of *FANCM* is inherited from the mother, while the large genomic deletion leading to a frame shift by skipping of exon 15 sequence is not found in blood DNA from the father and probably arisen in the germ line of the father. The splice site mutation in *FANCA* (IVS7+5G>A) is inherited from the mother, the nonsense mutation (R853X) is inherited from the father. EUFA867 has one affected brother (subject II:3) who carries the two *FANCA* mutations and only one of the *FANCM* mutations. This patient was first diagnosed as a typical FA patient. The phenotype of this patient seems more severe than that of EUFA867.



Supplementary Figure S3.3

Stable expression of FANCA in EUFA867 lymphoblasts partially corrects the cellular phenotype. (A) Stable transfection of EUFA867 lymphoblasts with FANCA cDNA does not restore MMC hypersensitivity. EUFA867 cells were stably transfected with episomal vector pMEP4 encoding FANCA-flag. Lymphoblasts were continuously exposed to different doses MMC and cell growth was compared to untreated cells. Wild type (HSC93) and FANCA deficient (HSC72) lymphoblasts are included as controls. (B) Stable transfection of EUFA867 lymphoblasts with FANCA cDNA partially corrects FANCD2 monoubiquitination. Wild type lymphoblasts (HSC93), untransfected EUFA867 lymphoblasts (EUFA867) and FANCA transfected EUFA867 lymphoblasts (EUFA867+FANCA) were treated for 16 hours with 5 µM aphidicolin, 320 µM HU or 200 nM MMC. Whole cell extracts were investigated for FANCD2 monubiquitination and FANCA expression by western blotting.



Supplementary Figure S3.4

Camptothecin sensitivity in lymphoblastoid cell lines from different FA complementation groups. (A) Lymphoblasts with a defect in FANCA are as sensitive to growth inhibition with different doses camptothecin as wild type lymphoblasts. (B) Lymphoblasts with a defect in FANCB or FANCJ are as sensitive to growth inhibition with different doses camptothecin as wild type lymphoblasts, while lymphoblasts with a defect in BRCA2 are as sensitive as EUFA867 cells. (C) Lymphoblasts with a defect in FANCC and cell fusions between FANCC deficient cells and EUFA867 are as sensitive to growth inhibition as wild type lymphoblasts. (D) Lymphoblasts with a defect in FANCD2 are as sensitive to growth inhibition with different doses camptothecin as wild type lymphoblasts. (E) Lymphoblasts with a defect in FANCE are as sensitive to growth inhibition with different doses camptothecin as functionally corrected FA-E lymphoblasts (EUFA410+FANCE). Cell line EUFA410 was functionally corrected by stable transfection with vector pIRESneo encoding HA-tagged FANCE. (F) Lymphoblasts with a defect in FANCG are as sensitive to growth inhibition with different doses camptothecin as wild type lymphoblasts. (G) Lymphoblasts with a defect in FANCI are as sensitive to growth inhibition with different doses camptothecin as wild type lymphoblasts. (H) Lymphoblasts with a defect in FANCL are as sensitive to growth inhibition with different doses camptothecin as wild type lymphoblasts.

Chapter 4

Fancm-deficient mice reveal unique features of Fanconi anemia complementation group M

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Abstract

The Fanconi anemia (FA) core complex member FANCM remodels synthetic replication forks and recombination intermediates. Thus far, only one FA patient with FANCM mutations has been described, but the relevance of these mutations for the FA phenotype is uncertain. To provide further experimental access to the FA-M complementation group we have generated *Fancm*-deficient mice by deleting exon 2. FANCM deficiency caused hypogonadism in mice and hypersensitivity to cross-linking agents in MEFs, thus phenocopying other FA mouse models. However, $Fancm^{\Delta 2/\Delta 2}$ mice also showed unique features atypical for FA mice, including underrepresentation of female $Fancm^{\Delta 2/\Delta 2}$ mice and decreased overall and tumor-free survival. This increased cancer incidence may be correlated to the role of FANCM in the suppression of spontaneous sister chromatid exchanges as observed in MEFs. In addition, FANCM appeared to have a stimulatory rather than essential role in FANCD2 monoubiquitination. The FA-M mouse model presented here suggests that FANCM functions both inside and outside the FA core complex to maintain genome stability and to prevent tumorigenesis.

Introduction

Fanconi anemia (FA) is a recessively inherited genetic disorder characterized by developmental abnormalities, progressive bone marrow failure and cancer susceptibility [1]. To date, 13 complementation groups have been defined (FA-A, B, C, D1, D2, E, F, G, I, J, L, M, N) and the corresponding genes have been identified [2]. At the cellular level, FA is characterized by hypersensitivity to cross-linking agents, a hallmark that is exploited in the clinic as a diagnostic test for FA [3]. Therefore, all FA proteins are thought to function in a pathway which mediates cross-linker resistance and maintains genomic stability [4]. The FA pathway is commonly divided in an upstream and a downstream part. The upstream part is involved in the monoubiquitination of FANCD2 and FANCI after DNA damage and during S phase [1,5-7]. This monoubiquitination is catalyzed by the FA core complex consisting of 8 FA proteins, FANCA, -B, -C, -E, -F, -G, -L, -M, and two FA- Associated Proteins FAAP100 and FAAP24 [8-10]. FANCL is the E3 ubiquitin ligase of the FA core complex [6], which is assisted by the E2 enzyme UBE2T [11]. Monoubiquitinated FANCD2 and FANCI are targeted to the chromatin where they co-localize with proteins involved in DNA repair, such as RAD51 [12]. Subsequently, the FA pathway appears to merge with the BRCA1/BRCA2 pathway, since hypomorphic mutations in *BRCA2* have been found in FA-D1 patients, and mutations in *PALB2*, encoding a partner and stabilizer of BRCA2, have been found in FA-N patients. Moreover, mutations in the gene encoding the BRCA1-interacting protein BRIP1 are found in the FA-J complementation group [13-15]. BRCA2/FANCD1, BRIP1/FANCJ, and PALB2/FANCN are supposed to act in the downstream part of the FA pathway, since the monoubiquitination of FANCD2 is not affected in patients carrying a defect in these proteins.

Although many components of the FA/BRCA pathway have now been identified, the precise role of this pathway in genomic maintenance remains unclear. Further eluci-

dation of the FA pathway is in part hampered by the fact that most FA core complex members are orphan proteins that cannot be connected to any known protein family based on sequence homology [16]. In this respect FANCM is an exception since it is not only an ortholog of the archaeal DNA repair protein HEF, but also contains two conserved domains: a helicase domain at the N-terminus and a (degenerate) endonuclease domain at the C-terminus [17,18]. FANCM is assigned to the helicase superfamily 2 and contains seven helicase-specific motifs (I, Ia II, III, IV, V, and VI) that are essential for its helicase function [19]. Motif II, the Walker B motif, consists of a DEAD or DEAH box and is necessary for the interaction with Mg2+ and thus for ATP hydrolysis. FANCM was shown to have translocase activity and to promote branch migration of Holliday junctions and replication forks [17,20,21] and could provide a link between the FA core complex and DNA cross-link repair.

FANCM was initially identified as FAAP250, a 250 kDa FA-Associated Protein that co-immunoprecipitated with other FA core complex members using an antibody against FANCA [9]. The classification of FAAP250 as the FA protein FANCM was based on lack of FAAP250 expression and the presence of biallelic mutations in patient EUFA867. This patient had been excluded from other existing complementation groups based on linkage analysis, cell fusion and cDNA transfection and was therefore assigned to the new reference group FA-M [17]. To date only one FA-M patient has been described, from whom only lymphoblasts are available for *in vitro* studies. In this FA-M reference cell line monoubiquitinated FANCD2 was absent and the levels of FANCA and FANCG were reduced [17]. Recently it has become apparent that this patient also carriers *FANCA* mutations, which questioned the actual contribution of FANCM deficiency to the FA phenotype [22].

Several FA mouse models have been reported to date: two for *Fancc* [23,24], two for *Fanca* [25,26], two for *Fancg* [27,28], one for *Fancl/POG* (proliferation of germ cells) [6,29], and one for *Fancd*2 [30]. None of these FA mice displayed the developmental abnormalities that are frequently observed in FA patients, such as missing digits or radii. However, gonadal abnormalities and reduced fertility, which are common in FA patients, have been observed in all FA mouse models. Consistently, ovaries in FA mice contained fewer developing follicles, while the seminiferous tubules in the testes were heterogeneous. Some tubules showed normal spermatogenesis, while others were devoid of germ cells and showed no evidence for active spermatogenesis [23,25,27,31,32]. In contrast to FA patients, none of the FA mice developed bone marrow failure or acute myeloid leukaemia. Only administration of sublethal doses mitomycin C (MMC) induced hematopoietic failure in FA mice [33]. In addition, in some FA mice, microphthalmia and perinatal lethality were observed [30,34]. Similar to cells from FA patients, cells derived from FA mouse models were hypersensitive to DNA cross-linking agents, as manifested by reduced survival, increased chromosomal breakage and G2 arrest. Another hallmark of FA core complex defects that was recapitulated in mice was the absence of FANCD2 monoubiquitination [23-28,28].

We generated a *Fancm* mouse model to study the effect of *Fancm* deficiency in vivo and observed that less female *Fancm* mice were born than expected. Moreover, *Fancm*

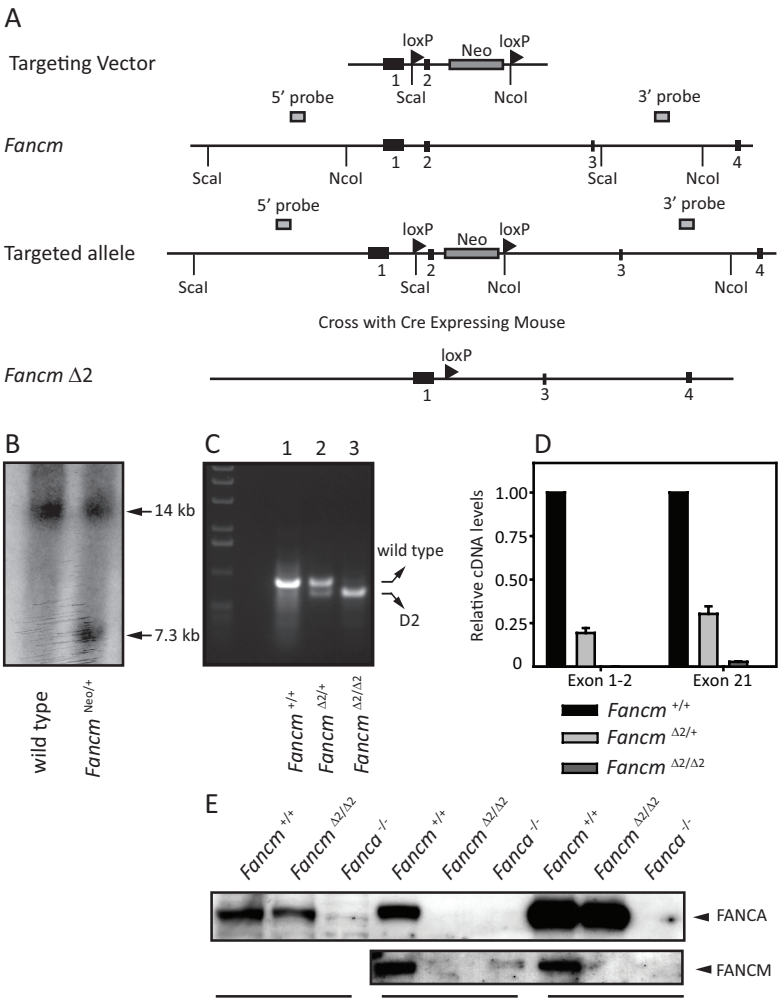

FIGURE 4.1

Disruption of the murine *Fancm* gene. (A) Schematic representation of the *Fancm* targeting vector, the wild type *Fancm* gene on chromosome 11, the targeted allele and the resulting *Fancm* Δ2 allele. Homologous recombination between the targeting vector and the endogenous *Fancm* sequence inserts a Neo cassette flanked by LoxP sites. The 5' and 3' probes are indicated by bars. As indicated, Cre-mediated recombination between LoxP sites leads to deletion of exon 2 and the *Neo* marker. (B) Southern blot analysis using genomic DNA from targeted ES cells digested with *ScaI* and hybridized with the 5' probe. Integration of the targeting vector introduces an additional *ScaI* site leading to a targeted band of 7.3 kb besides the wild type band of 14.0 kb. (C) PCR products of *Fancm* wild type, heterozygous or homozygous MEFs. *Fancm*-specific cDNA was produced with a primer in exon 10. This cDNA was subsequently amplified with primers in exon 1 and 10. Lane 1 contains the PCR product from $Fancm^{+/+}$ MEFs, lane 2 from $Fancm^{\Delta 2/+}$ MEFs and lane 3 from $Fancm^{\Delta 2/\Delta 2}$ MEFs. (D) Quantitative PCR on cDNA produced with oligo d(T)20VN primers from $Fancm^{+/+}$, heterozygous $Fancm^{\Delta 2/+}$ or homozygous $Fancm^{\Delta 2/\Delta 2}$ MEFs. *Fancm*-specific cDNA was amplified with primers in exon 1 and exon 2 and primers in exon 21. (E) Whole-cell lysates of MEFs of the indicated genotypes were immunoprecipitated with an antibody against human FANCM or murine FANCA, separated by SDS-PAGE and probed with an antibody against murine FANCA and human FANCM.

TABLE 4.1
Offspring of *Fancm* $^{\Delta 2/+}$ intercrosses

	Male		Female	
	Observed	Expected	Observed	Expected[a]
Fancm $^{\Delta 2/\Delta 2}$	18	22	6	22
Fancm $^{\Delta 2/+}$	50	43	44	43
Fancm $^{+/+}$	19	22	25	22
Total	87		75	
P-value	0.32		0.002*	

*Significant deviation from expected Mendelian frequency.
aBased on the number of male animals born.

mice showed reduced overall and tumor-free survival. The increased cancer incidence may be due to increased genetic instability as observed in FANCM-deficient mouse embryonic fibroblasts (MEFs).

RESULTS

Generation of a *Fancm* mouse model

Fancm knockout mice were generated by targeted deletion of exon 2, encoding amino acids 158 to 215 including the DEAH-box. This deletion does not only inactivate the helicase activity of FANCM, but also leads to a frameshift and premature stop in exon 3. A targeting construct was used that integrates a neomycin resistance cassette into intron 2 of the *Fancm* gene and LoxP sites in introns 1 and 2 flanking exon 2 and the Neo cassette (Figure 4.1A). Mice carrying this *Fancm* mutant allele were obtained (Figure 4.1B) and crossed with a Cre recombinase deleter mouse to remove exon 2 and the Neo marker. Since this *Fancm* mouse model was created by deleting exon 2 we will refer to it as the *Fancm*$^{\Delta 2}$ model. *Fancm*-specific cDNA was produced with a primer in exon 10 and subsequently amplified with primers in exon 1 and 10. In heterozygous *Fancm*$^{\Delta 2/+}$ MEFs, amplification of the wild type allele (Figure 4.1C, lane 2, upper band) was more prominent than that of the knock-out allele (lane 2, lower band). Although this was not a quantitative assay, these results suggested that the mRNA corresponding to the knock-out allele was less stable than mRNA transcribed from the wild type allele. Sequence analysis of the PCR products confirmed the loss of exon 2 from the *Fancm*Δ2 allele (data not shown). Quantitative RT-PCR (qPCR) with primers in exon 1 and exon 2 also demonstrated the loss of exon 2 sequence from the *Fancm* cDNA, since no product was formed and qPCR with primers in exon 21 showed barely detectable levels of *Fancm* cDNA in a *Fancm* mutant MEF cell line (Figure 4.1D). These data confirm that *Fancm* expression was lost in our *Fancm* mouse model, probably due to nonsense-mediated decay of *Fancm* mRNA. By immunoprecipitation and Western blotting with different antiserea against human FANCM we could demonstrate that FANCM protein was indeed absent in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs (Figure 4.1E) Furthermore, while FANCM co-immunoprecipitated with FANCA in *Fancm*$^{+/+}$ MEFs, FANCM was

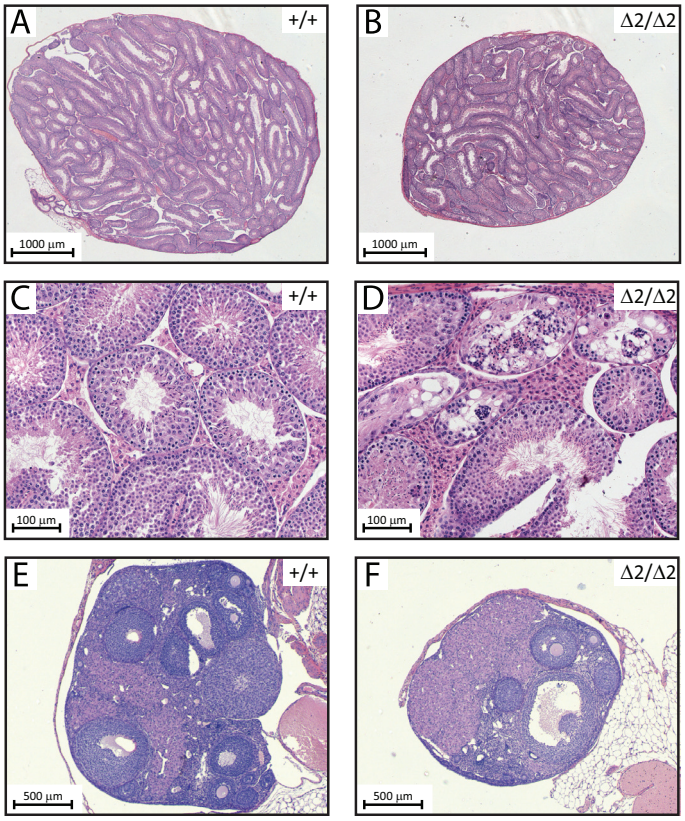

FIGURE 4.2
Gonadal abnormalities of *Fancm*$^{\Delta 2/\Delta 2}$ mice. Histological sections of testes (A-D) and ovaries (E-F) from 3-months old mice. (A) Control testis (magnification 2.5x). (B) *Fancm*$^{\Delta 2/\Delta 2}$ testis (2.5x). (C) Control testis (25x). (D) *Fancm*$^{\Delta 2/\Delta 2}$ testis (25x). (E) Control ovary (5x). (F) *Fancm*$^{\Delta 2/\Delta 2}$ ovary (5x).

absent in a FANCA immunoprecipitate in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs. These results showed that no full length FANCM protein was expressed in the *Fancm*$^{\Delta 2/\Delta 2}$ cells.

Non-Mendelian inheritance of *Fancm*$^{\Delta 2/\Delta 2}$ alleles in females

The *Fancm* targeting was performed in the Bruce4 ES cell line and cells were injected into C57/Bl/6J blastocysts. The chimeras were backcrossed to the C57/Bl/6 and subsequently backcrossed to the FVB mouse strain. *Fancm*$^{\Delta 2/+}$ mice from backcross 2 and 3 were inter-crossed to obtain *Fancm*$^{\Delta 2/\Delta 2}$ offspring. Male *Fancm*$^{\Delta 2/\Delta 2}$ mice were born with the expected Mendelian frequency. However, a significant decrease in the number of female *Fancm*$^{\Delta 2/\Delta 2}$ mice was observed (Table 4.1). Up till now, an underrepresentation of female mice has not been reported for FA mouse models. At present, we are unable to explain the decrease in the number of female *Fancm*$^{\Delta 2/\Delta 2}$ mice. Since genetic background might play a critical role we are crossing the *Fancm*$^{\Delta 2/+}$ mice into different mouse strains.

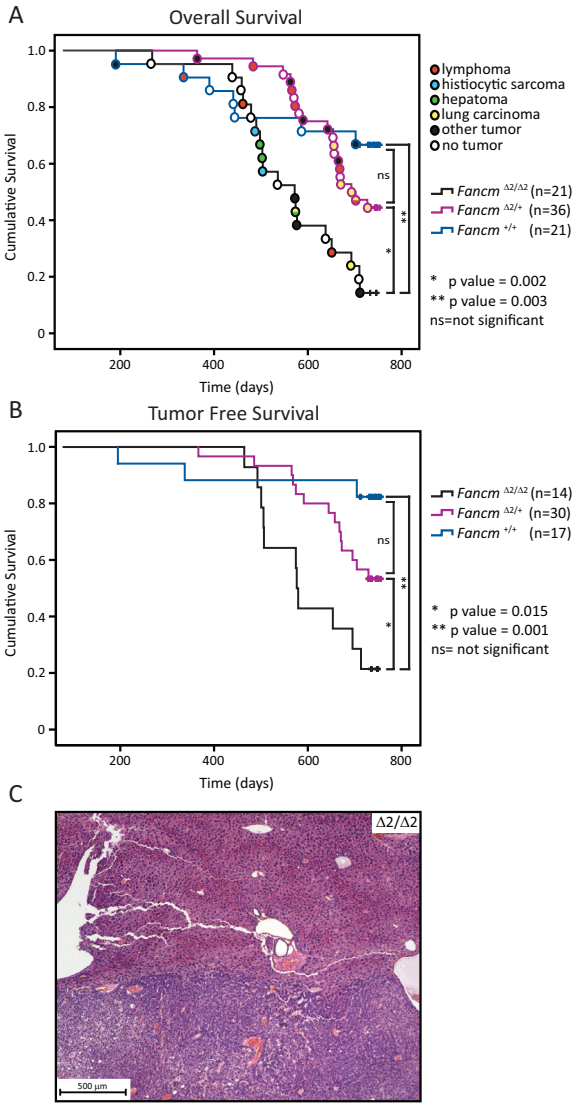

FIGURE 4.3

Kaplan Meier survival curves of *Fancm*$^{\Delta 2/\Delta 2}$ mice and control littermates. (A) Overall survival curves for *Fancm*$^{\Delta 2/\Delta 2}$ (black line), *Fancm*$^{\Delta 2/+}$ (purple line) and *Fancm*$^{+/+}$ (blue line) mice. Tumor types detected in the individual animals are indicated by coloured circles. (B) Tumor-free survival curves for *Fancm*$^{\Delta 2/\Delta 2}$ (black line), *Fancm*$^{\Delta 2/+}$ (purple line) and *Fancm*$^{+/+}$ (blue line) mice. (C) Representative picture of a hepatoma from a *Fancm*$^{\Delta 2/\Delta 2}$ mouse (magnification 5X).

Fancm$^{\Delta 2/\Delta 2}$ mice show gonadal abnormalities

One phenotype shared by all FA mouse models is the appearance of gonadal abnormalities associated with reduced fertility [23-28,30,35]. We performed histology on the reproductive organs of 3-months old male and female *Fancm*$^{\Delta 2/\Delta 2}$ mice. The testicular size in *Fancm*$^{\Delta 2/\Delta 2}$ males was reduced compared to wild type littermates (Figure 4.2A, B). The seminiferous tubules of the *Fancm*$^{\Delta 2/\Delta 2}$ males were heterogeneous: some tubules showed an absence of spermatogonia and did not contain sperm (Figure 4.2D), while others had normal testicular architecture and sperm production. In addition, *Fancm*$^{\Delta 2/\Delta 2}$ testes showed hyperplasia of Leydig cells. This pattern was not present in wild type littermates (Figure 4.2C). We have not thoroughly investigated whether these gonadal abnormalities also affected fertility, but noticed that several male mice had been able to produce offspring. The number of developing follicles in *Fancm*$^{\Delta 2/\Delta 2}$ ovaries was reduced in comparison to wild type ovaries and the cortex of *Fancm*$^{\Delta 2/\Delta 2}$ ovary was depleted of primary follicles (Figure 4.2E, F). Due to the low number of *Fancm*$^{\Delta 2/\Delta 2}$ females, we have not investigated their fertility. These results demonstrate that *Fancm*$^{\Delta 2/\Delta 2}$ mice phenocopy other FA mouse models with respect to gonadal abnormalities

Fancm$^{\Delta 2/\Delta 2}$ mice show reduced life span and increased tumor formation

We did not observe congenital abnormalities in the *Fancm*$^{\Delta 2/\Delta 2}$ mice. Also the bone marrow of *Fancm*$^{\Delta 2/\Delta 2}$ mice showed no signs of hypoplasia and the number of hematopoietic stem cells appeared normal (data not shown).

To monitor the effect of FANCM deficiency on survival we followed cohorts of 21 *Fancm*$^{\Delta 2/\Delta 2}$ (16 males + 5 females), 36 *Fancm*$^{\Delta 2/+}$ (22 males + 14 females) and 21 *Fancm* $^{+/+}$ (8 males + 13 females) mice for two years and plotted the overall survival of this cohort in a Kaplan Meier curve (Figure 4.3A). The median survival of *Fancm*$^{\Delta 2/\Delta 2}$ mice (573 days) was significantly reduced in comparison to *Fancm*$^{\Delta 2/+}$ (693 days, p-value=0.002) and *Fancm*$^{+/+}$ (>730 days, p-value=0.003) littermates. The median survival of *Fancm*$^{\Delta 2/\Delta 2}$ females (491 days) and *Fancm*$^{\Delta 2/\Delta 2}$ males (573 days) did not significantly differ and also male and female heterozygous littermates had a similar lifespan. We noted that of the *Fancm*$^{\Delta 2/\Delta 2}$ mice, 52% (11 out of 21) developed tumors whereas 39% of the *Fancm*$^{\Delta 2/+}$ (14 out of 36) and 14% of the *Fancm*$^{+/+}$ (3 out of 21) mice developed tumors. Therefore we hypothesized that the *Fancm*$^{\Delta 2/\Delta 2}$ mice have an increased incidence of tumor formation. To follow tumor-free survival we calculated a Kaplan Meier curve that excluded the mice sacrificed because of non-tumor related pathology (Figure 4.3B). We observed a statistically significant decrease in tumor-free survival in the *Fancm*$^{\Delta 2/\Delta 2}$ mice compared to the *Fancm*$^{\Delta 2/+}$ (p-value= 0.015) and *Fancm*$^{+/+}$ (p-value=0.001) littermates. We noted several different tumors in our cohort of which one tumor type, hepatoma, was detected in three *Fancm*$^{\Delta 2/\Delta 2}$ mice as opposed to zero hepatomas in 57 control mice (p-value = 0.013). A picture of one of these hepatomas is shown (Figure 4.3C) Apart from an increased tumor incidence we also observed arteritis nodosa in 3 out of 21 *Fancm*$^{\Delta 2/\Delta 2}$ mice compared to 1 in 57 control animals (p-value = 0.013). This may be indicative of a premature aging phenotype in *Fancm*$^{\Delta 2/\Delta 2}$ mice.

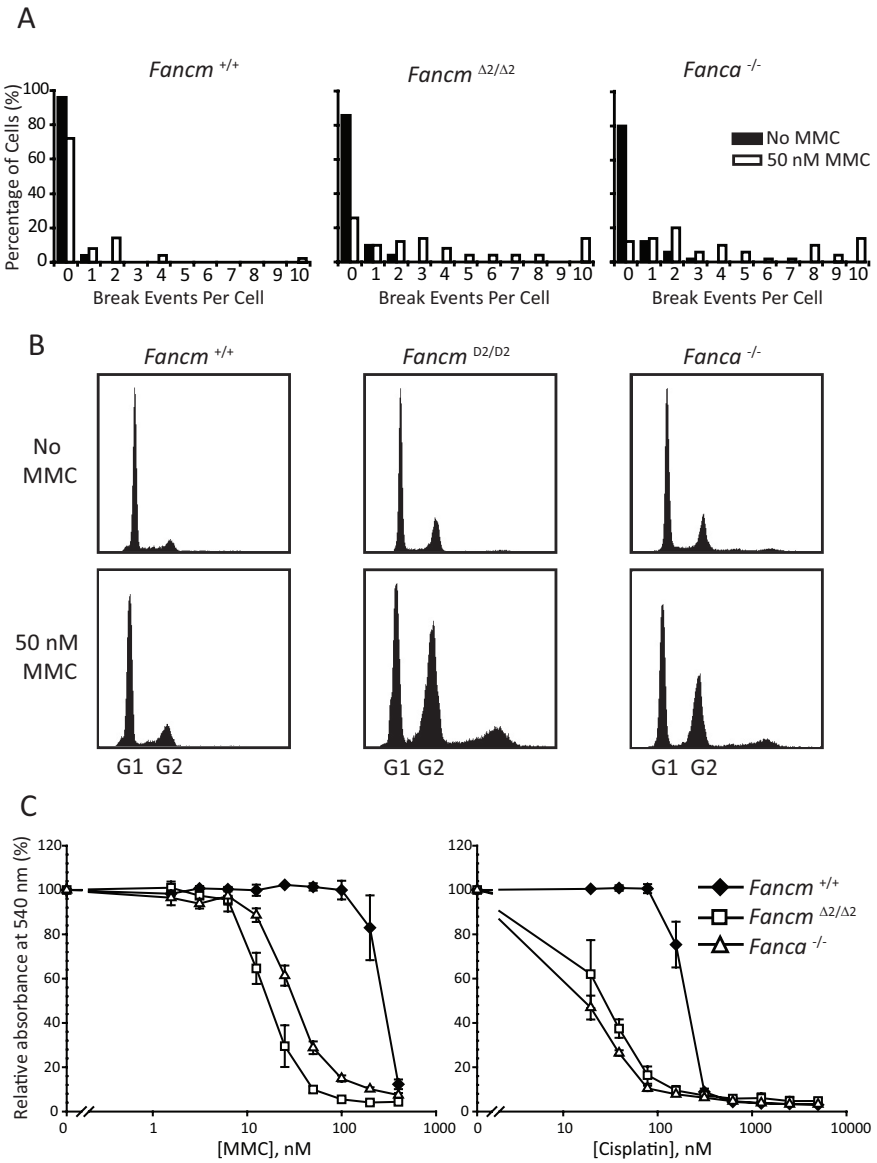

FIGURE 4.4

Cellular phenotype of *Fancm*$^{\Delta 2/\Delta 2}$ MEFs. (A) *Fancm*$^{\Delta 2/\Delta 2}$ MEFs and *Fanca*$^{-/-}$ MEFs showed markedly elevated chromosomal breakage after MMC treatment (50 nM for 48h, white bars) compared to wild type MEFs. (B) *Fancm*$^{\Delta 2/\Delta 2}$ MEFs showed a marked accumulation in the G2 phase of the cell cycle after MMC treatment (50 nM for 72h) similar to the response of *Fanca*$^{-/-}$ MEFs, whereas a G2 arrest was not apparent in control *Fancm*$^{+/+}$ MEFs. (C) *Fancm*$^{\Delta 2/\Delta 2}$ MEFs (squares) and *Fanca*$^{-/-}$ MEFs (triangles) showed reduced colony survival after continuous exposure to MMC or cisplatin when compared to *Fancm*$^{+/+}$ MEFs (diamonds).

In summary, we observed an increased cancer incidence and associated reduced life span in our *Fancm*$^{\Delta 2/\Delta 2}$ cohort when compared to heterozygous and wild type littermates. To investigate whether the increased cancer incidence could be attributed to increased genetic instability, we studied the consequences of *Fancm* deficiency in MEFs.

Fancm$^{\Delta 2/\Delta 2}$ MEFs show an FA-like phenotype

A hallmark of FA cells is a pronounced hypersensitivity to cross-linking drugs such as MMC resulting in increased chromosomal breakage, reduced cell viability and a pronounced G2 arrest. We assessed this phenotype in immortalized MEF cell lines that were either deficient or proficient for the FANCM protein. Immortalized *Fanca*$^{-/-}$ MEFs were used as an FA control [25]. The number of chromosomal breaks and chromosomal aberrations was more strongly increased by MMC treatment in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs and *Fanca*$^{-/-}$ MEFs than in *Fancm*$^{+/+}$ MEFs (Figure 4.4A).

Another feature of FA cells is a prolonged G2 arrest after exposure to cross-linking agents [36-38]. Following incubation with MMC, *Fancm*$^{\Delta 2/\Delta 2}$ MEFs showed a pronounced G2 arrest, which was also apparent in *Fanca*$^{-/-}$ MEFs, but was absent in *Fancm*$^{+/+}$ MEFs (Figure 4.4B). The G2 arrest was already noticed after 24 hours (Supplementary Figure S4.1) and did not correspond to an arrest in mitosis since the cells stained negative for the mitotic marker MPM2. In fact, a decrease in the mitotic fraction was observed that is consistent with an arrest in G2 (data not shown). In both *Fanca*$^{-/-}$ and *Fancm*$^{\Delta 2/\Delta 2}$ MEFs, we observed cells with a DNA content higher than 4N. This >4N population was likely caused by cells that had completed mitosis but failed cytokinesis and had started to synthesize DNA. As shown in figure 4A, some of the *Fancm* and *Fanca* deficient MEFs had escaped from G2 arrest and entered mitosis in the presence of chromosomal aberrations. Since these MEFs were immortalized with E1A they lacked the G1 checkpoint and therefore these 4N cells could start a new round of DNA replication resulting in a >4N peak. Correspondingly, this >4N population was absent in primary MEFs (Supplementary Figure S4.2).

Next, we assessed whether FANCM deficiency resulted in reduced growth after exposure to MMC or cisplatin. As shown in Figure 4.4C, *Fancm*$^{\Delta 2/\Delta 2}$ MEFs and *Fanca*$^{-/-}$ MEFs were hypersensitive to MMC and cisplatin when compared to *Fancm*$^{+/+}$ MEFs. Thus far, all these assays confirmed that *Fancm*$^{\Delta 2/\Delta 2}$ MEFs exhibited a cellular phenotype resembling that of *Fanca*$^{-/-}$ MEFs and characteristic for FA cells.

Fancm$^{\Delta 2/\Delta 2}$ MEFs are able to monoubiquitinate FANCD2

The FA core complex acts as an E3-ubiquitin ligase that monoubiquitinates FANCD2 and FANCI. A distinctive feature of cells deficient in any of the FA core complex members is their inability to monoubiquitinate FANCD2 and FANCI during S phase or after replicative stress and DNA damage [5,7,39]. FANCM is an integral component of the FA core complex and based on the results obtained with the FA-M reference cell line EUFA867 it was concluded that FANCM was essential for FA core complex formation and FANCD2 monoubiquitination [17]. Transient knockdown of FANCM

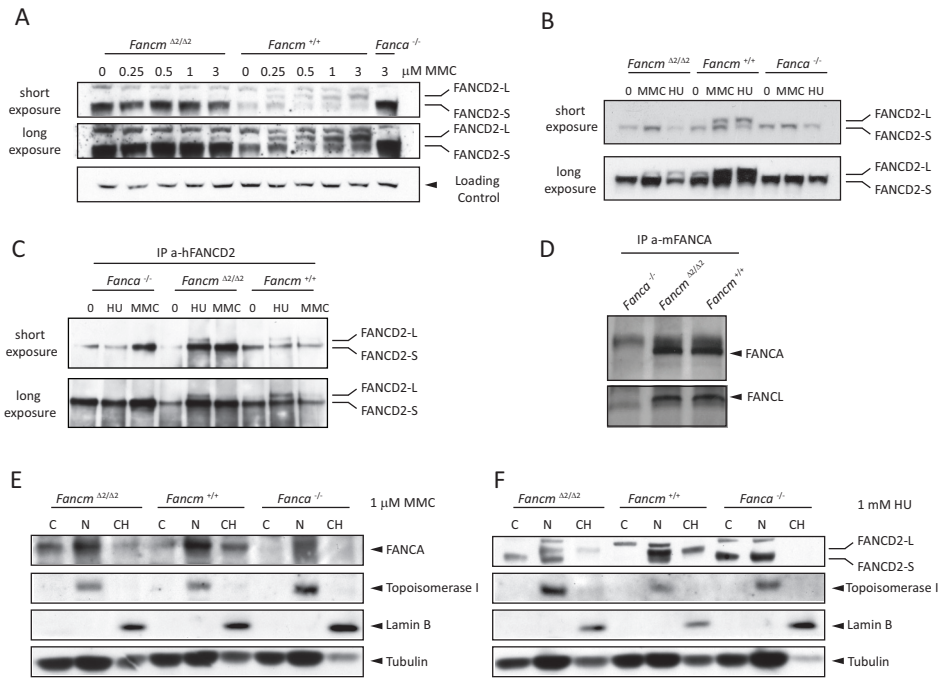

FIGURE 4.5

Fancm$^{\Delta 2/\Delta 2}$ MEFs show FANCD2 monoubiquitination. (A) MEFs of the indicated genotypes were either left untreated or treated with different doses MMC for 24h. Whole-cell lysates were separated by SDS-PAGE and probed with an antibody against murine FANCD2. (B) Cells were treated with MMC (3 μM for 24h) or with HU (1mM for 24h). Whole-cell lysates were separated by SDS-PAGE and probed with an antibody against murine FANCD2. (C) Cells were treated with 0.5 μM MMC or 1 mM HU for 24h and cell lysates were immunoprecipitated with an antibody against human FANCD2, separated by SDS-PAGE and probed with another antibody against human FANCD2. (D) Whole-cell lysates of MEFs of the indicated genotypes were immunoprecipitated with an antibody against murine FANCA, separated by SDS-PAGE and probed with an antibody against murine FANCA and human FANCL. (E) MEFs of the indicated genotypes were treated with 1μM MMC and fractionated. Fraction were separated by SDS-PAGE and probed with an antibody against murine FANCA. C indicates the cytoplasmic, N, the nuclear and CH the chromatin fraction. As control for the fractionation procedure antibodies against γ-tubulin (cytoplasmic), topoisomerase I (nuclear) and Lamin B (chromatin) were used. (F) MEFs of the indicated genotypes were treated with 1 mM HU and fractionated. Fraction were separated by SDS-PAGE and probed with an antibody against murine FANCD2.

protein using siRNA confirmed the necessity of FANCM for normal FA core complex function [40,41]. To investigate whether FANCM is indeed essential for FANCD2 monoubiquitination, lysates from untreated MEFs or MEFs treated with different doses of MMC were probed with a FANCD2 antibody. FANCD2 monoubiquitination is seen as a shift from a short non-monoubiquitinated isoform (FANCD2-S) to a longer monoubiquitinated isoform (FANCD2-L). Western blot analysis revealed the presence of both the short and long isoforms of FANCD2 in the *Fancm*$^{+/+}$ MEFs after MMC treatment (Figure 4.5A). In contrast, the FANCD2-L isoform appeared absent in cell lysates from *Fanca*$^{-/-}$ and *Fancm*$^{\Delta 2/\Delta 2}$ MEFs. Upon extended exposure of the Western blot, however, the FANCD2-L isoform became visible in lysates from MMC-treated *Fancm*$^{\Delta 2/\Delta 2}$ MEFs, but still remained undetectable in lysates from *Fanca*$^{-/-}$ MEFs. This indicated that in the absence of FANCM, FANCD2 could still be monoubiquitinated in a MMC-dose dependent way, albeit at strongly reduced levels. We also found that in response to HU FANCD2 was monoubiquitinated in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs (Figure 4.5B). To verify this unexpected finding we performed an immunoprecipitation with a human FANCD2 antibody and probed the blot with a different FANCD2 antibody. Again, residual FANCD2 monoubiquitination was observed in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs, but not in *Fanca*$^{-/-}$ MEFs (Figure 4.5C). These results suggest that the formation of a functional FA core complex was not critically dependent on FANCM, which was strengthened by the co-precipitation of FANCA and FANCL in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs (Figure 4.5D). A further indication for a role of the FA core complex in FANCD2 monoubiquitination in the absence of FANCM was given by chromatin fractionations. As shown in figure 5E, FANCA was still enriched in the chromatin fraction after MMC treatment in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs, albeit at reduced level in comparison to wild-type MEFs. Furthermore, similar to the situation in wild type MEFs part of the monoubiquitinated FANCD2 was retained in the chromatin fraction in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs (Figure 4.5F). These results indicated that in the absence of FANCM part of the core complex can still localize to the chromatin and monoubiquitinate FANCD2.

Primary *Fancm*$^{\Delta 2/\Delta 2}$ MEFs show increased sister chromatid exchanges

In chicken DT40 cells deficient for the chicken homologue of FANCM, Hef, increased levels of sister chromatid exchanges (SCEs) were reported [42,43]. We scored SCE frequencies in two independent *Fancm*$^{\Delta 2/\Delta 2}$ primary MEF cultures and in heterozygous and wild type *Fancm* primary MEFs. The basal SCE level in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs was significantly increased (p-value < 0.0001) in comparison to *Fancm*$^{\Delta 2/+}$ and *Fancm*$^{+/+}$ MEFs (Figure 4.6A). The increased spontaneous SCE frequency is specific for *Fancm*$^{\Delta 2/\Delta 2}$ MEFs since two independent primary *Fanca*-deficient MEF cultures had similar SCE frequencies than wild-type MEFs (Figure 4.6B). We also tested the efficiency of SCE induction after DNA damage. To that end, replication-associated double strand breaks (DSB) were induced by N-methyl-N'-nitrosoguanidine (MNNG), an alkylating agent that produces single-strand breaks (SSB) in the first replication cycle resulting in a replication-associated DSB in the second cell cycle. As shown in Figure 4.6A, the SCE frequency increased after MNNG exposure, but this increase was similar between the different genotypes (p-value = 0.103). We conclude that *Fancm*$^{\Delta 2/\Delta 2}$ MEFs have a significant elevated basal level of SCEs, as was observed in the chicken DT40 system

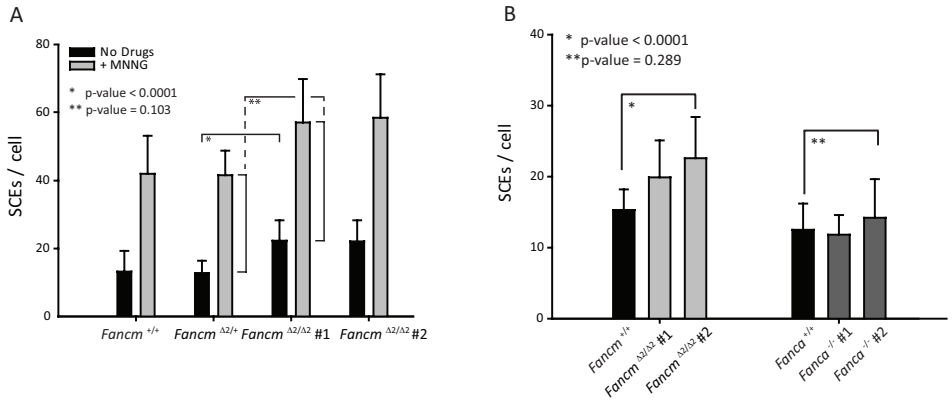

FIGURE 4.6

Increased SCEs in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs. (A) Primary MEF cultures (one *Fancm*$^{+/+}$, one *Fancm*$^{\Delta 2/+}$, two different *Fancm*$^{\Delta 2/\Delta 2}$) were labelled with BrdU (6 µg/ml) during 48h in the absence or presence of MNNG (6 µM) plus O6-BG (20 µM). SCEs were counted in 20 second-division metaphase spreads. (B) Primary MEF cultures (one *Fancm*$^{+/+}$, two different *Fancm*$^{\Delta 2/\Delta 2}$, one *Fanca*$^{+/+}$ and two different *Fanca*$^{-/-}$ MEF cell lines) were labelled with BrdU (6 µg/ml) during 48h. SCEs were counted in 20 second-division metaphase spreads. Data show the mean SCE frequencies per metaphase and error bars indicate standard deviations. Statistical significance was calculated with the Mann Whitney test (SPSS, V15).

[44]. However, the induction of SCE frequency after the generation of DSBs seems independent of FANCM.

Discussion

FANCM is the first FA core complex protein in which conserved domains have been identified that may point to an enzymatic role in DNA repair. Moreover, recent studies showed that FANCM can promote branch migration of Holliday junctions and DNA replication structures [20,21,45], suggesting that FANCM may provide a link between the FA pathway and DNA repair. The *Fancm* mouse model reported here supports this concept. At first glance, *Fancm*-deficient mice resembled mice deficient for other FA core complex members: they showed gonadal abnormalities and at the cellular level they displayed increased chromosomal breakage, excessive G2 arrest, and crosslinker hypersensitivity. However, four additional atypical features were observed: non-Mendelian segregation of *Fancm*$^{\Delta 2/\Delta 2}$ alleles in females, decreased overall and tumor-free survival, residual FANCD2 monoubiquitination and increased spontaneous SCEs.

Fancm knockout mice were born at a decreased Mendelian ratio, which was specifically due to an underrepresentation of female *Fancm*$^{\Delta 2/\Delta 2}$ mice. In contrast, normal Mendelian ratios were reported for most other FA mouse models. [23-28]. Perinatal lethality was observed in one of the *Fancc* mouse models and in the *Fancd2* mouse model, both in a C57Bl/6 background, but these studies did not report on differences

in gender distribution [30,34]. In the *Fancl/POG* model, embryonic lethality was seen in a pure 129/Sv background and in the F1 generation of a cross between 129/Sv and C57Bl/6. However, normal Mendelian ratios were observed in F1 offspring from heterozygous crossings between 129/Sv and FVB [46]. Female embryonic lethality has been described in mice nullizygous for both *Msh2* and *p53* in a mixed C57Bl/6 and 129/Sv background, but this was overcome in crosses with BALB/c and SWR/J mice [47,48]. In summary, the genetic background affects the viability of knockout mice suggesting the presence of modifier loci that, when combined with an FA defect, result in reduced viability. We are currently crossing the *Fancm*$^{\Delta 2/+}$ mice into the C57Bl/6 strain to investigate whether the underrepresentation of female *Fancm*$^{\Delta 2/\Delta 2}$ is modified by the genetic background of the mice.

An important difference between the *Fancm*$^{\Delta 2/\Delta 2}$ mice and other FA defective mice (*Fanca*, *Fancc*, *Fancd2*, *Fancg*, and POG/*Fancl*) is the reduced life span and increased cancer incidence in the homozygous *Fancm* mice compared to littermates. For two FA mouse models, the *Fancc* and *Fancd2* deficient mice, long term survival cohorts have been published. In the *Fancc*-defective mice no tumors were observed after 600 days and only in combination with loss of tumor suppressor *Trp53* (*Trp53*$^{+/-}$ or *Trp53*$^{-/-}$) *Fancc* loss resulted in a shortened latency of tumorigenesis [49]. For the *Fancd2* defective mice a statistically significant increase in the incidence of both adenomas and carcinomas was reported [30], but in a follow-up study of the same group this difference was not considered significant [50]. Again, only the combination of *Trp53*$^{+/-}$ with *Fancd2* loss resulted in a significant decrease in tumor-free survival [51]. The decreased overall and tumor-free survival observed in the *Fancm*$^{\Delta 2/\Delta 2}$ mice therefore appears unique for this FA mouse model and suggests that FANCM has additional functions independent of the FA core complex in the prevention of tumor formation.

This additional function may be reflected by the elevated spontaneous sister chromatid exchanges in primary *Fancm*$^{\Delta 2/\Delta 2}$ MEFs. Like in *Fancm* deficient MEFs, disruption of FANCM in the chicken B cell line DT40 resulted in an increased background level of SCEs [52,53]. This phenotype is shared with cells from Bloom's Syndrome patients, which are deficient for BLM, an anti-recombinogenic RecQ helicase. The BLM protein acts as a tumor-suppressor gene by suppressing recombination and thereby reducing loss of heterozygosity (LOH) through mitotic recombination [54]. The helicase domain of FANCM is shared with yeast orthologs MPH1 (S. cerevisiae) and FML1 (S. pombe), that play a regulatory role in homologous recombination repair by replication fork reversal and D-loop disruption [55-57]. Possibly human and mouse FANCM have a similar role. The basal increase in SCE frequency may indicate that FANCM prevents the formation of DSBs after replication fork stalling and may be a reflection of the increased damage at the replication fork. It has been shown that the FANCM protein can remodel replication fork substrates [20,21]. Possibly in the absence of FANCM stalled replication forks are not correctly processed leading to collapsed replication forks and DSBs. These DSBs may be repaired via various repair pathways, some of which result in SCEs [19]. Analogous to cells deficient for the BLM protein this elevated frequency of spontaneous SCEs may result in an increased rate of LOH and could explain the increased cancer incidence observed in the *Fancm*$^{\Delta 2/\Delta 2}$ mice.

However, FANCM seems to have no function in the outcome of the recombination process as supported by the lack of an additional significant increase in SCE frequency upon MNNG treatment, which induces DSBs, when compared to control MEFs.

Residual FANCD2 monoubiquitination was detectable in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs. This indicates that in the absence of FANCM a functional FA core complex can still be formed, a conclusion supported by the co-precipitation of FANCA and FANCL in *Fancm*$^{\Delta 2/\Delta 2}$ MEFs and enrichment of FANCA in the chromatin fraction. Possibly, FANCM has a role in recruiting the FA core complex to the site of damage and positions this complex in the vicinity of FANCD2 for efficient monoubiquitination, as suggested by siRNA experiments in HeLa cells (36). These findings seem to be in contrast with data published on the FA-M lymphoblasts (EUFA867). In these cells, immunoblotting showed no evidence for FANCD2 monoubiquitination, reduced levels of FANCA and FANCG and defective nuclear localization of FANCA and FANCL. This discrepancy between the human FA-M lymphoblasts and murine *Fancm*$^{\Delta 2/\Delta 2}$ MEFs can however be explained by the presence of biallelic FANCA mutations in the EUFA867 cells, which implies that the human reference cell line is deficient for both FANCM and FANCA [58]. Therefore, our knockout mice and MEFs represent the first mammalian model system in which the role of FANCM can be investigated in a defined genetic background with littermate controls.

In summary, we have shown that FANCM deficiency is associated with an FA phenotype in mice and MEFs. The system also revealed several unique features related to FANCM deficiency, including decreased overall survival, increased cancer incidence and female perinatal lethality in mice, and residual FANCD2 monoubiquitination and increased SCEs in MEFs. These novel phenotypes show that FANCM has an important role in the prevention of tumorigenesis and suggest that FANCM partially acts independent of the FA core complex to maintain genome stability.

Materials And Methods

ES cell targeting and generation of *Fancm*$^{\Delta 2/\Delta 2}$ mice

Targeting and production of mice was performed at Ozgene (Australia). The *Fancm* targeting vector was linearized and electroporated into Bruce4 ES cells. After selection with G418, genomic DNA from surviving clones was digested with *ScaI* and *NcoI* and screened by Southern blotting with a 5' probe and 3' probe, respectively (primers for 5' probe: TTGAAATGGTCCAAGTCAGGTCA and GATAGCGAGTGCTGTGTGATTCC and primers for 3' probe: TGGGGTGTTTTCTGCTCTGACC and ATGTCTGCCATTCTCCT GCGGG). Correctly targeted ES cell clones were injected into C57BL/6J blastocysts. Chimeric mice were crossed with a B6 deleter mice expressing Cre recombinase, to recombine the LoxP sites and remove exon 2 and the selection marker. The *Fancm*$^{\Delta 2/+}$ mice were then backcrossed to the FVB strain. Homozygotes were produced by matings of BC2 and BC3 heterozygous mice.

Generation of cDNA, sequence analysis and quantitative PCR

Total RNA was isolated from *Fancm*$^{+/+}$, *Fancm*$^{\Delta 2/+}$ and *Fancm*$^{\Delta 2/\Delta 2}$ MEFs using RNA-Bee Total RNA Isolation Reagent (Campro Scientific). cDNA was prepared by reverse transcription using a primer in exon 10 (primer in exon 10: 5'-CCACCGTCTCG-GAACTG-3'). From this template, a product was amplified by PCR using primers in exon 1 (primer in exon 1: 5'-TTCATTGCCGCCGTGGTCA-3') and exon 10. This PCR product was cloned into the pGEM®-T Easy vector (Promega). Vector primer T7 was used for sequencing. For quantitative PCR (qPCR), 1 µg of total RNA was used to prepare cDNA by reverse transcription using oligodN6 random primers (Roche Diagnostics). Subsequently, the cDNA was used as a template for qPCR in the presence of SYBR-green (Applied Biosystems) to label the product. Fluorescence detection was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All measurements were performed in triplicate and the relative amounts of cDNA were compared to actin as a reference for total cDNA. Subsequently, the *Fancm*$^{+/+}$ cDNA concentration was used for normalization. For qPCR of exon 1-2 we used 5'-GCTGAAATGACAGGTTCAACTC-3' as forward and 5'-CATTTACCATGACCT-GCGG-3' as reverse primer, for qPCR of exon 21 we used 5'-TGCTTCACCACCATG-TACTGG-3' as forward and 5'-AGCCACATGCAGGAGTGAAGT-3' as reverse primer.

Histological analysis

Mice were killed and isolated organs were fixed in formol. The testes and ovaries of mutant and wild type mice were fixed in EAF fixative (ethanol–acetic acid–formol saline). Fixed organs were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Kaplan Meier Survival Curves

Overall and tumor-free survival curves were generated using SPSS software (Version 15), statistical significance between genotypes was determined using built-in analysis for survival curves consisting of a log rank test yielding a p-value.

Isolation, culturing and transduction of MEFs

MEFs were derived from 13-day-old embryos and cultured in GMEM (Invitrogen-GIBCO) supplemented with 10% fetal calf serum, 1 mM non-essential amino acids (Invitrogen-GIBCO), 10 mM sodium pyruvate (Invitrogen-GIBCO), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen-GIBCO), and 0.1 mM β-mercaptoethanol (Merck) at 37°C, under a humidified atmosphere of air plus 5% CO2. MEFs were immortalized by viral infection with E1A. Ecotropic retroviral supernatants were produced by transfecting Phoenix cells by calcium phosphate co-precipitation. Thirty-six hours post-transfection, retroviral supernatants were filtered through a 0.45 µm filter (MCE membrane, Millipore) and used to infect MEFs. MEFs were transduced in the presence of 4 µg/ml polybrene.

MMC-induced chromosomal breakage analysis

MEFs immortalized with E1A were cultured for 48 h in the absence or presence of 50 nM MMC. For each cell culture, 50 metaphases were analyzed for chromatid-type chromosomal abnormalities. To quantify chromosomal abnormalities the interchange aberrations were converted into break events. All scoring was performed blind to eliminate counting bias [59].

Growth inhibition assays.

E1A immortalized MEFs were seeded in 96-well microplates on day 0 in Dulbecco's modified Eagle's medium with glutamate (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) and sodium pyruvate (1mM) and supplied with 2-fold serial drug dilutions on day 1. On day 7, the cells were fixed by adding trichloroacetic acid (Merck) to a final concentration of 5% (v/v). After 1h at 4°C, plates were washed five times with demi water, dried, and stained for 30 min with 50 µL sulforhodamine B (0.4%, w/v) (Sigma). Following three wash steps with 1% acetic acid, 200 µL 10 mM Tris was added to dissolve the staining. Absorbance at 540 nm was measured using a Tecan infinite m200 plate reader (Tecan). After correction for medium-only and no-drug controls, data points were fitted using the general formula for a sigmoid curve.

G2 arrest

Cells were seeded in Petri dishes at 2.5 * 105 cells and allowed to attach overnight. Cells were exposed to either 50 or 100 nM MMC and were grown for 24h, 48h or 72h. Cells were trypsinized and fixed in 70% ethanol at 4°C for at least 1h. Subsequently, cells were washed in PBS and nuclei were stained with propidium iodide (4 µg/ml) and treated with RNAse (2 µg/ml) in 200 µl PBS, incubated at 37 °C for 15 min, and analyzed in a flowcytometer using FACS 'Cell Quest' software and 'Summit' software.

Immunoblot and immunoprecipitation analysis

For preparation of whole-cell extracts, cells were lysed for 30 min on ice in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 0.1%SDS supplemented with 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml). For immunoblot analysis equivalents of 200,000 cells were loaded on a 3-8% Tris-Acetate NuPAGE gradient gel (Invitrogen) and proteins were separated by gel-electrophoresis at 25 mA during 3.5 h, according to the manufacturer's protocol. Proteins were transferred to Immobilon-P-Transfer membranes (Millipore) and the membranes were blocked with 5% dry milk in PBS-Tween (0.1%). The membranes were incubated with the indicated antibodies. After washing with PBS-Tween (0.1%), the membranes were incubated with horseradish peroxidase-conjugated secondary antbodies (DakoCytomation). Proteins were visualized with ECL. For immunoprecipitation reactions, cells were lysed for 30 min on ice in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40 supplemented with 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml), and incubated with

either guinea pig antibody against human FANCD2 (amino acids 55-292), guinea pig antiserum against murine FANCA or guinea pig antiserum against human FANCM [60] and 50% protein A beads (Pharmacia), overnight at 4°C, under gentle agitation. Prior to immunoblot analysis, lysates were washed five times in lysis buffer after which immunoblot analysis was performed as described above. Antibodies used for immunoblot analysis were rabbit polyclonal antiserum against murine FANCD2 (gift from Dr. A.D. D'Andrea)[61], rabbit polyclonal antiserum against human FANCD2 (gift from Dr. K.J. Patel), rabbit polyclonal antiserum against murine FANCA [6,62]), rabbit polyclonal against antiserum against human FANCL and rabbit polyclonal against antiserum against human FANCM (gifts from Dr. W. Wang)[6].

Chromatin Fractionations

The chromatin fractionation was essentially performed as in [63]. Briefly, cells were collected by trypsinization and pelleted. The pellet was resuspended and incubated for 10 min in ice-cold buffer containing 10 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.2% triton and complete protease inhibitor cocktail (Roche). The suspension was homogenized and the supernatant containing the cytoplasmic fraction was collected after 15-min centrifugation at 400 x g at 4 °C. The pellet was washed in ice-cold PBS, resuspended in cold buffer containing 420 mM NaCl, 20 mM Hepes-KOH (pH 7.9), 20% glycerol, 2 mM MgCl2, 0.2 mM EDTA, 0.1% triton, 0.5 mM DTT, and complete protease inhibitor cocktail (Roche) and incubated on ice for 1 h. The supernatant containing the nuclear fraction was collected after 15-min centrifugation at 18,000 x g at 4 °C. The chromatin/membrane-containing pellet was resuspended in cold PBS supplemented with 600 mM NaCl, 1% N-octyl glucoside, and 125 units of DNAse, incubated for 30 min in an ultrasonic bath; and centrifuged for 15 min at 18,000 x g at 4 °C. Chromatin/membrane proteins were collected with the supernatant. 1,3 mg of protein of each fraction were separated on a 3-¬8% Tris-Acetate NuPAGE gradient gel (Invitrogen). As control for the fractionation procedure antibodies against γ-tubulin (cytoplasmic, Sigma), topoisomerase I (nuclear) and Lamin B (chromatin, Santa Cruz) were used.

Sister chromatid exchanges (SCEs)

To experimentally induce SCEs we chose to mimic a replication-associated DSB with N-methyl-N'-nitrosoguanidine (MNNG) (Serva, Heidelberg, Germany). The alkylating agent MNNG produces single-strand gaps in the first replication cycle that, if left unrepaired due to the presence of O6-methylguanine-DNA methyltransferase (MGMT) inhibitor O6-benzylguanine, results in a replication-associated DSB[64] . Cycling cells were labeled for 48h with 8 µg/ml 5'bromodeoxyuridine (BrdU). Exposure time to MNNG (6 µM) and O6-benzylguanine (20 µM) was 48h. The metaphase spreads were essentially prepared as described [65] . Briefly, slides were stained with bisBenzimide H33258 (Sigma B-2883), illuminated with UV for 1h, heated for 90 min in 2 x SCC at 65o C, followed by staining in 10% Giemsa solution (Merck 1.09204). Twenty metaphases were analyzed for the presence of SCEs.

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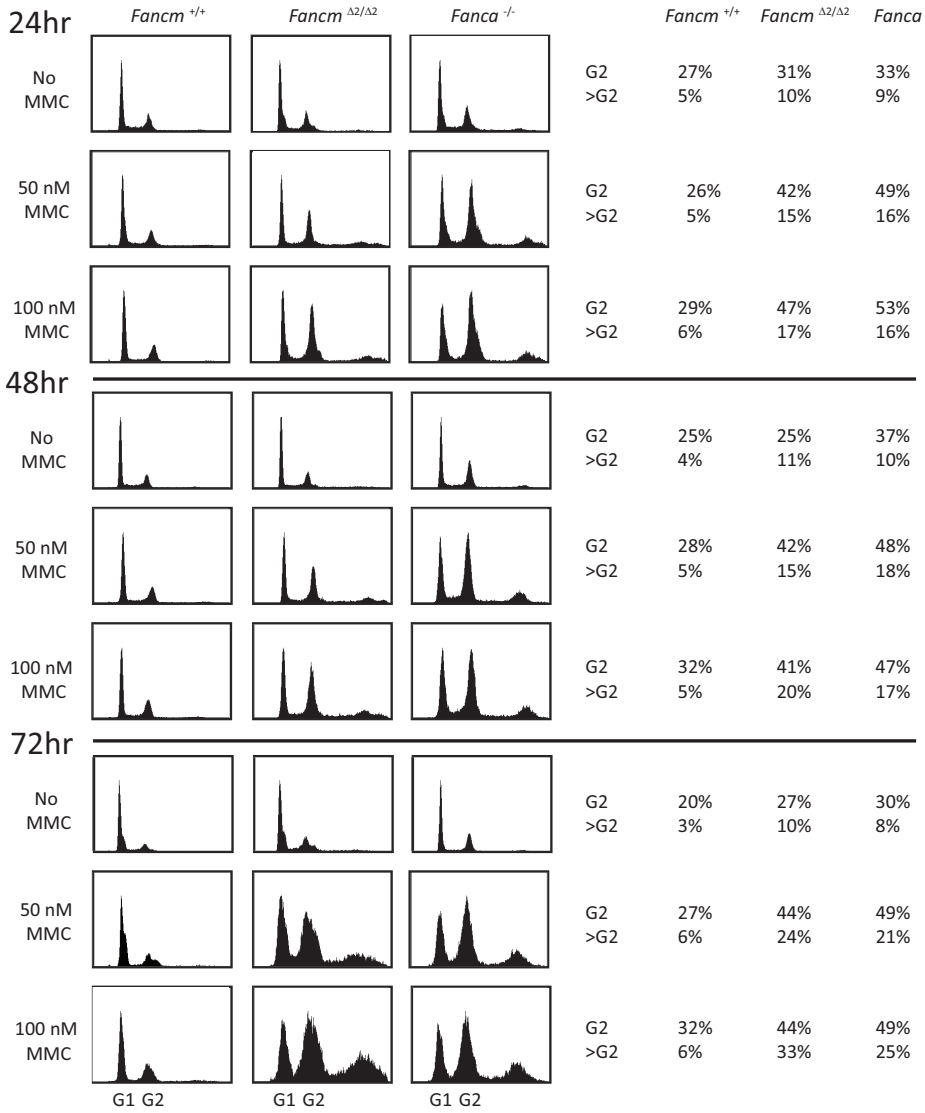
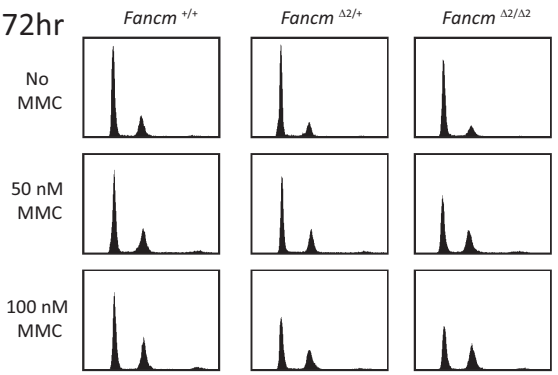
Supplementary Figures



SUPPLEMENTARY FIGURE S4.1

Time course of the accumulation in the G2 phase of the cell cycle after MMC treatment. *Fancm*$^{\Delta 2/\Delta 2}$, *Fancm*$^{+/+}$ and *Fanca*$^{-/-}$ MEFs were treated with either 50 or 100 nM MMC for 24h, 48h or 72 h. The percentages of cells in the G2 phase of the cell cycle and the >4N population were quantified for each MMC dosage and time point.



		Fancm $^{+/+}$	*Fancm* $^{\Delta2/+}$	*Fancm* $^{\Delta2/\Delta2}$
No MMC	G2	26%	21%	20%
	>G2	5%	4%	3%
50 nM MMC	G2	32%	31%	38%
	>G2	6%	5%	5%
100 nM MMC	G2	35%	36%	42%
	>G2	8%	7%	6%

Supplementary Figure S4.2

Time course of the accumulation in the G2 phase of the cell cycle after MMC treatment. Primary *Fancm*$^{\Delta2/\Delta2}$, *Fancm*$^{\Delta2/+}$ and *Fancm*$^{+/+}$ MEF cultures were treated with either 50 or 100nM MMC for 72 h. The percentages of cells in the G2 phase of the cell cycles and the >4N population were quantified for each MMC dosage and time point.

Chapter 5

Fancf-deficient mice are prone to develop ovarian tumors

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Abstract

Fanconi Anemia (FA) is a rare recessive disorder marked by developmental abnormalities, bone marrow failure, and a high risk for the development of leukemia and solid tumors. The inactivation of FA genes, in particular FANCF, has also been documented in sporadic tumors in non-FA patients. To study whether there is a causal relationship between FA pathway defects and tumor development, we have generated a mouse model with a targeted disruption of the FA core complex gene *Fancf*.

Fancf-deficient mouse embryonic fibroblasts displayed a phenotype typical for FA cells: they showed an aberrant response to DNA crosslinking agents as manifested by G2 arrest, chromosomal aberrations, reduced survival and an inability to monoubiquitinate FANCD2.

Fancf homozygous mice were viable, born following a normal Mendelian distribution and showed no growth retardation or developmental abnormalities. The gonads of *Fancf* mutant mice functioned abnormally showing compromised follicle development and spermatogenesis as has been observed in other FA mouse models and in FA patients. In a cohort of *Fancf*-deficient mice, we observed decreased overall survival and increased tumor incidence. Notably, in 7 female mice 6 ovarian tumors developed, 5 granulosa cell tumors and one luteoma. One mouse had developed tumors in both ovaries. High-resolution array comparative genomic hybridization (aCGH) on these tumors suggest that the increased incidence of ovarian tumors correlates with the infertility in *Fancf*-deficient mice and the genomic instability characteristic for FA pathway deficiency.

Introduction

Fanconi Anemia (FA) is a genetically and phenotypically heterogeneous disorder that is subdivided into at least 15 different complementation groups caused by mutations in 15 different genes [1-5]. Despite the genetic heterogeneity of this disease, all FA patients share several phenotypic characteristics: FA patients are predisposed to hematological malignancies, solid tumors of the head and neck region, and gynecological tumors. In addition, cells of FA patients show an increased number of chromosomal breaks after treatment with DNA crosslinking agents [6]. Based on this shared phenotype, all FA gene products are thought to function together in the FA pathway that is essential for repair of DNA crosslinks.

The FA pathway can be subdivided in different components. The upstream component consists of the FA core complex, which can monoubiquitinate FANCD2 and FANCI. The FA core complex consists of 8 proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM), and four FA associated proteins (FAAP24, FAAP100, MHF1, and MHF2)[7]. The downstream component consists of the FA proteins FANCD1, FANCJ, FANCN, FANCO and FANCP which are though to coordinate repair of DNA crosslinks by the combined action of several different repair pathways [1,3].

It is clear that a disrupted FA pathway results in an increased cancer risk in FA patients, but the significance of an inactivated FA pathway in sporadic tumors remains unclear. FANCF has generated much interest in this respect, since epigenetic silencing of FANCF has been reported in several different tumors such as ovarian[8], bladder [9], cervical [10], leukemic [11], testicular [12], lung, and oral tumors [13]. Especially the correlation between FANCF silencing and ovarian cancer has been reported repeatedly. Three independent studies have observed FANCF promoter hypermethylation in ovarian cancer cell lines and in ovarian cancer samples [8,14,15]. FANCF promoter methylation was also detected in ovarian granulosa cell tumors [16]. In addition, FANCF promoter hypermethylation was correlated with gene silencing and cisplatin sensitivity [8]. It has been hypothesized that epigenetic silencing of FANCF could be an early event in tumor aetiology, since it could result in genetic instability and drive the generation of additional mutations [3,8]. The reversible nature of epigenetic silencing could also explain initial sensitivity (FANCF silencing) and acquired resistance (FANCF re-expression) to crosslinking agents observed in ovarian cancer treatment [3,8,17]. Other studies, however, have not been able to confirm FANCF promoter methylation in tumors [18,19]. Therefore, the importance of FANCF silencing in sporadic tumor development is still under debate.

To date, several FA mouse models have been generated [20]. With the exception of *Fancp/Slx4* [21], none of these models displayed the FA-characteristic developmental abnormalities, but all mice showed gonadal abnormalities and infertility. Recent data demonstrate that the FA phenotype could be recapitulated when *Fancd2* deficiency was combined with aldehyde dehydrogenases 2 (ALDH2) deficiency, an enzyme responsible for the detoxification of aldehydes. These mice were only viable in the presence of fetal or maternal ALDH2 and succumbed to bone marrow failure when exposed to ethanol, an exogenous source of acetaldehydes [22]. Moreover the unexposed double homozygous animals died within 3 to 6 months due to acute lymphoid leukemia (ALL). These results suggest that the FA pathway is critical for counteracting aldehyde toxicity. Similar to cells from FA patients, cells derived from FA mouse models were hypersensitive to DNA crosslinking agents and cells with FA core complex defects were fully [20] or severely compromised in FANCD2 monoubiquitination [23].

To study the role of FANCF in the FA pathway and tumorigenesis, we generated *Fancf*-deficient mice. This mouse model confirmed that FANCF is an essential FA core complex member in mice and a survival cohort demonstrated that *Fancf* homozygous mice had a decreased tumor-free survival.

Materials And Methods

Cloning of murine *Fancf*

The single-exon *Fancf* coding sequence was identified in a C57BL6j mouse genomic λ phage library using a Genbank EST-based probe. *Fancf* DNA was amplified using primers CACGAGGTCCCTACACAGATGGAGGACATG and AGCCTGGGAACTGAGAATCTACTCTAGCAC. A 1551 bp *HindIII EcoRI* fragment encompassing the *Fancf*

coding sequence was cloned into pBluescript SK- and sequence verified. For functional analysis, the murine *Fancf* coding sequence was transferred to the mammalian episomal expression vector pMEP4. Patient-derived FA-F lymphoblastoid cells EUFA698 were stably transfected with pMEP4-*Fancf* and crosslinker resistance was assessed.

Construction of *Fancf* targeting vector

Using genomic DNA derived from 129Ola E14 ESC, 4 genomic *Fancf* sequences were obtained by Elongase (Invitrogen) and PCR products were cloned into pGEM-T Easy (Promega) and *Fancf* sequence identity was confirmed. Primer pairs are available upon request. The targeting vector was assembled on the loxP *Neomycin* selection cassette. The first *Fancf* sequence was cloned upstream of *Neomycin* in PacBac-His1 using *KpnI* and *XbaI*. The selection cassette and *Fancf* sequence were transferred using *XbaI* and *SalI* to *XhoI* into pBluescript SK-*Fancf* from which the 5' region of the ORF was deleted after double *XhoI* digestion and self ligation. The other 3 PCR fragments were inserted using *BstEII, BamHI, MfeI*, and *SpeI* sites within the genomic sequence. The complete Δ*Fancf* targeting construct was excised from the plasmid backbone using *KpnI* and *SpeI*.

Fancf targeting and ESC screening

The Δ*Fancf* targeting vector was transfected into 129Ola ESC and selected for G418 resistance. Resistant single cell-derived ESC clones were screened by PCR to identify correct recombination events upstream of the *Fancf* coding. DNA from PCR positive cells was further analyzed by Southern blotting in combination with *MscI* digestion applying the *Neo* ORF as probe. Moreover, a Southern blot was performed with genomic DNA in combination with a *SpeI* digest and a 3' probe downstream of the Δ*Fancf* targeting vector. The 3' probe was obtained by PCR on ESC genomic DNA using primers 8285mFscrFor (CAGCCCTCATCTAGTTTCTTTTC) and 9134mFscrRev (GCTCATCTGCTTTCCACTAC). Finally, the positive ESC clones were screened for 5' deletion of *Fancf* using PCR with primers *Neo*+248For (GCTCGACTGTGTCACTGAAG) and gF+236Rev (GACTCCAGCGCCGCGAAGC).

MEF isolation, culturing, and determining FA phenotype

MEFs were derived from 13-day-old embryos and cultured as described before [23]. The MMC induced chromosomal breakage analysis, growth inhibition assays, G2 arrests, and immunoblot assays were performed as described before [23].

Animal husbandry

Fancf mutant mice were maintained on an F1 between the FVB and 129Ola genetic background. Animals were examined regularly for tumor development and sacrificed if tumors were present or if animals were otherwise unhealthy. A final sacrifice of all animals occurred after 2 years. These mice are depicted as censored events in the survival plots. Overall and tumor-free survival curves were generated using SPSS software

(Version 17), statistical significance between genotypes was determined using built-in analysis for survival curves consisting of a log rank test yielding a p-value.

All animal experiments were approved by the local ethical review committee on animal experimentation and conducted according to Dutch legislation.

Histological analysis

Mice were sacrificed and isolated organs were fixed in formol. The testes and ovaries of mutant and wild type mice were fixed in EAF fixative (ethanol–acetic acid–formol saline). Fixed organs were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Slides were first scanned using a Scanscope XT digital slide scanner (Aperio, Vista, CA, USA) and further analyzed with Imagescope software (Aperio).

Follicle counting

Follicle count was performed as described previously using one ovary per animal [24]. Primordial follicles (diameter <20 µm), growing follicles, and corpora lutea were counted in every fifth section.

Gonadotropin levels

Blood was obtained from mice by retro-orbital collection using MiniCollectTM vials (Greiner Bio-One) and serum was isolated according to the manufacturer's protocol. By MSD/Organon (Oss, The Netherlands) gonadotropin levels were determined by an ELISA assay.

Array comparative genome hybridization (aCGH)

Genomic DNA was extracted from paraffin embedded tumors and spleens as previously described [25]. Methods of DNA labeling, array construction and hybridization, as well as methods for array normalization and data analysis have been described previously [26]. In brief, tumor and reference samples were fragmented by sonification and random-prime labeled with Cy3 and Cy5 dyes. Labeled material was co-hybridized to microarrays consisting of 135.000 oligonucleotide probes throughout the mouse genome (median probe spacing around 20 kb). Arrays were scanned at 532 nm (Cy3) and 635 nm (Cy5) using a microarray scanner and data were extracted using NimbleScan software. After normalization and log2-ratio calculation, copy number gains and losses were identified using the DNAcopy algorithm included in Nimblescan software. We used standard parameters for the segmentation.

Quantification of gains and losses from aCGH data

We used the segments as calculated by the DNAcopy algorithm in the Nimblescan software to quantify genomic aberrations. We excluded segments containing less than 10 probes to eliminate possible noise-related segments. We considered any segment

with a mean log2 intensity of > .1 or < -.1 to be a true CNA. For the correlation analysis we smoothed each tumor individually using the comparative module of the KCsmart R package available in the toolbox Bioconductor in the R statistical programming language [27]. We used standard parameters for the KCsmart smoothing (sigma = 1 Mb) and 1 minus the Pearson's correlation of the smoothed genomic profiles as a distance measure for hierarchical clustering using average linkage.

Results

Murine FANCF complements an FA-F patient cell line

To ascertain the functional conservation between murine and human FANCF, we made use of the patient-derived FA-F lymphoblastoid cell line EUFA698, which shows sensitivity to MMC in a growth-inhibition assay. As shown in Figure 5.1A, ectopic expression of murine FANCF restored MMC resistance in the FA-F patient cell line to the same extent as human FANCF cDNA. This indicates that murine and human FANCF proteins are functionally conserved.

Generation of a *Fancf* mouse model

We used conventional gene targeting to disrupt the murine *Fancf* allele. We generated a targeting vector in which a LoxP-flanked neomycin (neo) selection cassette was inserted into the genomic *Fancf* sequence deleting 165 nt of the promoter region plus 171 nt of the *Fancf* ORF including the start codon (Figure 5.1B). We initially screened G418-selected embryonic stem cell (ESC) colonies by PCR to identify homologous recombination events between the targeting construct and the *Fancf* locus. Site specific recombination and single copy integration of the targeting vector was confirmed in two independent ESC clones (F1 and F2) by Southern blotting using *MscI* restriction in combination with a neo probe (Figure 5.1C) and *SpeI* restriction in combination with the 3'-probe (Figure 5.1D). Chimaeric mice were created by blastocyst injection and subsequent breeding produced *Fancf* heterozygous animals. These animals were interbred to produce mouse embryonic fibroblasts (MEFs) of all three *Fancf* genotypes (Figure 5.1E).

Fancf $^{-/-}$ MEFs show an FA-like phenotype

We used MEFs to study the in vitro consequences of FANCF deficiency. We never observed any difference between *Fancf* heterozygous and wild type MEFs so considered both genotypes as controls. A hallmark of FA cells is a pronounced hypersensitivity to crosslinking drugs such as Mitomycin C (MMC), resulting in increased chromosomal breakage, pronounced G2 arrest, and reduced cell viability.

We first scored the number of chromosomal breaks and aberrations with or without MMC treatment, an assay that is also used for FA diagnosis in humans. As shown in Figure 5.2A *Fancf*-deficient primary MEF cultures (right panel) accumulated more break events after MMC treatment than control MEF cultures (left panel).

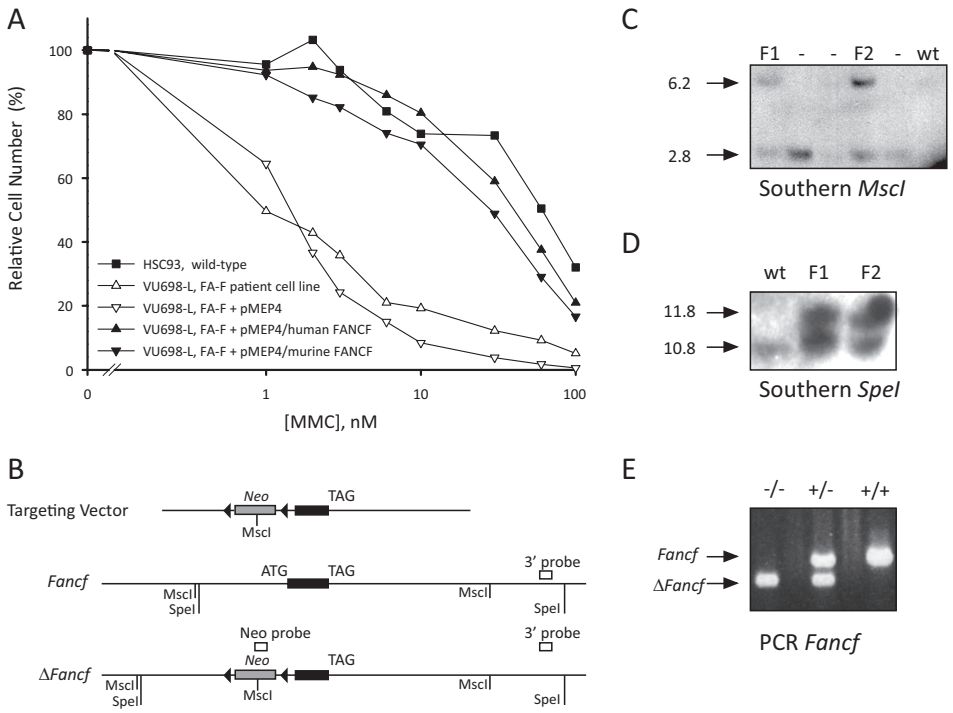


FIGURE 5.1

(A) Complementation of the MMC sensitivity in patient-derived FA-F cells by murine FANCF. *Fancf* DNA in the mammalian expression vector pMEP4 was stably transfected into the human FA-F lymphoblastoid cell line EUFA698 (△). In a growth inhibition test murine FANCF (▼) fully restored MMC sensitivity in EUFA698 cells to the level observed in wild-type cells, HSC93(■). A similar level of complementation was achieved by expressing human FANCF cDNA in EUFA698 (▲). pMEP4 mock-transfected (▽) and parental EUFA698 cell lines (△) exhibit the FA characteristic cross-linker sensitivity. (B) Schematic representation of the *Fancf* targeting strategy. A targeting vector was constructed in which a LoxP-flanked *Neomycin* selection cassette was inserted into a genomic *Fancf* sequence deleting 165 nt of the promoter region plus 171 nt of the *Fancf* ORF including the start codon. (C) To confirm site-specific recombination and single copy integration of the targeting vector, genomic DNA from G418-resistant ESC clones was digested with the *MscI* endonuclease. Following homologous recombination the *Neo* cassette will introduce an additional *MscI* recognition site in the *Fancf* locus resulting in restriction fragments of 2.8 and 6.2 Kb. Lanes indicated by – were not targeted correctly. Genomic DNA from ESC clones F-1 and F-2 showed the appropriately sized DNA fragments as detected by a *Neo* probe on Southern blot. (D) Homologous recombination between the targeting vector and the *Fancf* locus was further confirmed by Southern blot using a DNA probe 3' of the targeting vector in combination with digestion of genomic ESC DNA with *SpeI*. The targeting vector adds 1 kb to the endogenous *SpeI* fragment of the *Fancf* locus. After *SpeI* digestion of genomic DNA from ESC F-1 and F-2, two DNA bands of 10.8 and 11.8 kb were detected by the 3'-probe indicating the *Fancf* wild-type and targeted alleles. Digestion of genomic DNA from wild-type ESC only showed the 10.8 kb *SpeI* fragment (WT). (E) PCR products for FANCF from *Fancf* $^{-/-}$, *Fancf* $^{+/-}$, *Fancf* $^{+/+}$ MEFs confirms loss of the *Fancf* gene in the FANCF mouse model.

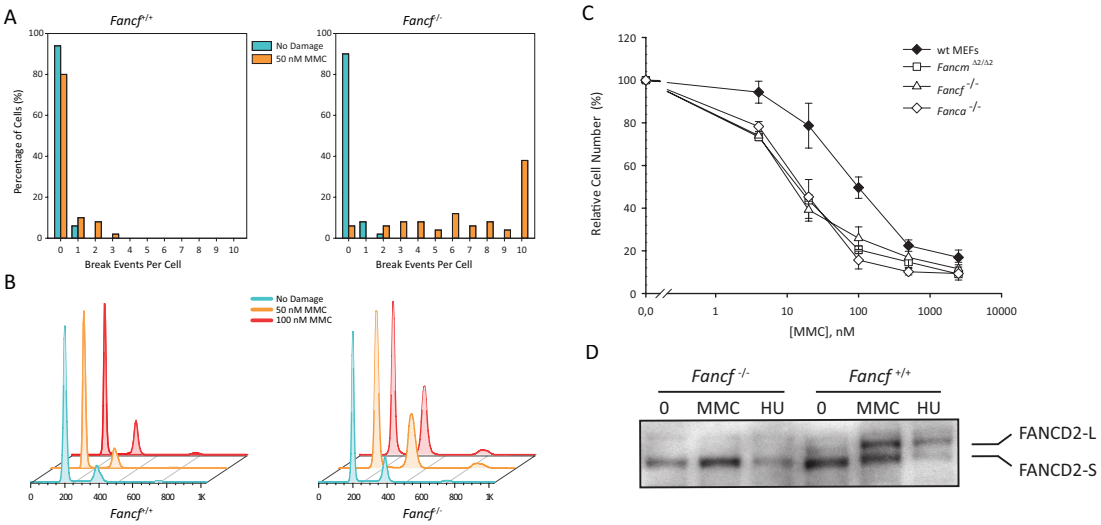


FIGURE 5.2

Fancf $^{-/-}$ MEFs have a disrupted FA pathway. (A) Chromosomal breakage in primary *Fancf* $^{-/-}$ and wild-type MEF cultures without treatment (blue bars) or after exposure to 50 nM MMC for 48h (orange bars) (B) Cell cycle analysis of primary *Fancf*$^{-/-}$ MEF and wild-type MEF cultures without damage or after MMC treatment (50 nM or 100 nM for 72h). (C) Growth inhibition assay after continuous exposure to MMC using *Fancf* $^{+/+}$ MEFs (diamonds), *Fancf* $^{-/-}$ MEFs (squares) and Fanca $^{-/-}$MEFs (triangles). (D) Western blot for FANCD2 in cell lysates from *Fancf* $^{-/-}$ and *Fancf* $^{+/+}$ MEFs after treatment with MMC (3 μM for 24h) or with hydroxyurea (HU, 1 mM for 24h).

Another feature of FA cells is a cell cycle arrest with 4N DNA content after exposure to crosslinking agents [28]. Primary *Fancf* $^{-/-}$ MEF cultures arrested at 4N after MMC treatment (Figure 5.2B, right panel) and this arrest was less pronounced in wild-type primary MEF cultures (Figure 5.2B, left panel). The *Fancf*-deficient MEF cultures also contained cells with a DNA content higher than 4N, suggesting that some cells had failed cytokinesis and entered another round of replication.

Next, we assessed whether *Fancf*-deficiency resulted in reduced proliferation after MMC treatment. As shown in Figure 5.2C, *Fancf* $^{-/-}$ MEFs were hypersensitive to the growth-inhibiting effect of MMC, just as Fanca- and Fancm-deficient MEFs [20,23]

The FA core complex acts as an E3-ubiquitin ligase that monoubiquitinates FANCD2 and FANCI during S phase and after DNA damage. To determine whether FANCF is essential for FANCD2 monoubiquitination, we performed a Western blot on equal numbers of cells that were unexposed or exposed to MMC or hydroxyurea. As shown in Figure 5.2D, both the short and long isoforms of FANCD2 were present in control MEFs, whereas in *Fancf* $^{-/-}$ MEFs only the short non-monoubiquitinated isoform (FANCD2-S) was present.

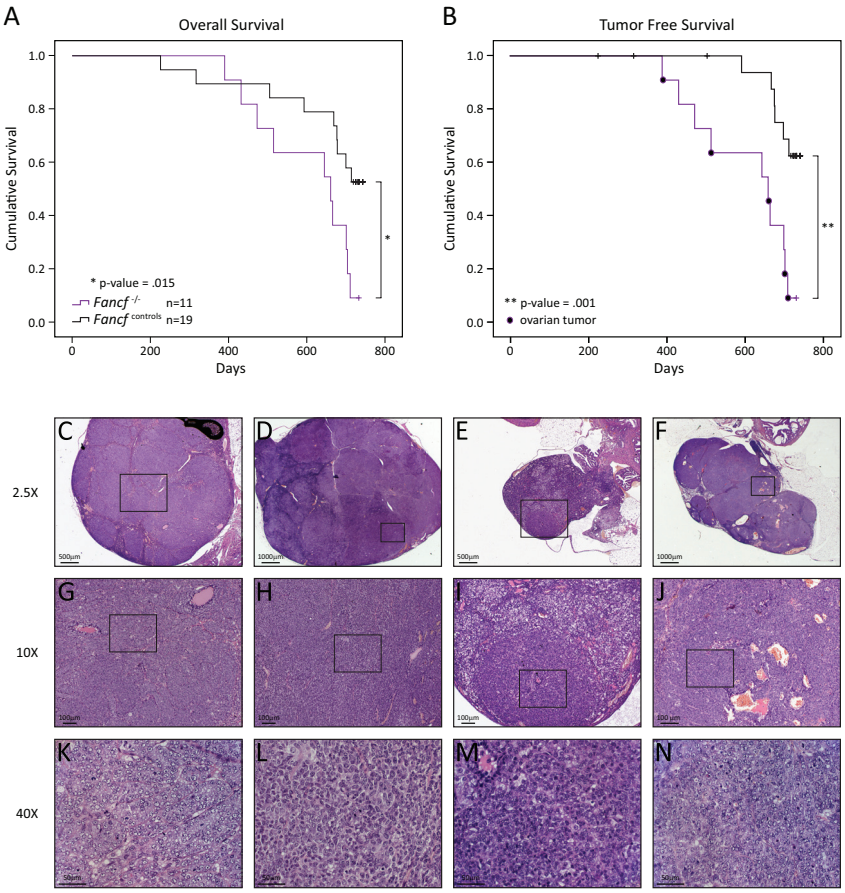


FIGURE 5.3

Kaplan Meier survival curves of *Fancf* $^{-/-}$ mice and control littermates. (A) Overall survival curves for *Fancf* $^{-/-}$ (purple line) and *Fancf* controls (black line) mice. (B) Tumor-free survival curves for *Fancf*$^{-/-}$ (purple line) and *Fancf* controls (black line) mice. The mice that developed ovarian tumors are depicted in the Kaplan-Meier curve in Fig. 3B as filled circles. (C-F) 2.5X magnification pictures of the affected ovaries with a granulosa cell tumor. (G-J) 10x magnifications and (K-N) 40X magnifications of the affected ovaries.

These data show that FANCF is an essential FA core complex member and in its absence the FA pathway is impaired, which results in the failure to maintain genome stability.

Fancf $^{-/-}$ mice are born at Mendelian ratio and show no developmental abnormalities

We intercrossed *Fancf* $^{+/-}$ mice to obtain *Fancf* $^{-/-}$ offspring. All gender and genotype combinations were present as expected from normal Mendelian distribution (data not shown). We carefully inspected the *Fancf*-deficient mice and found no overt developmental abnormalities. Since growth retardation is common in FA patients, we also weighed the mice at 3-months of age, but we did not note any differences (data not shown).

Fancf $^{-/-}$ mice are tumor prone

To investigate the consequence of *Fancf*-deficiency for overall survival, we followed cohorts of 11 *Fancf* $^{-/-}$ and 19 littermate control mice for two years. Animals were sacrificed when moribund and extensive necroscopy was performed. The Kaplan-Meier plot for overall survival is shown in Figure 5.3A. There was a significant difference (p-value = 0.015) between the mean overall survival of *Fancf* $^{-/-}$ mice (603 days) and control animals (658 days). We noted that *Fancf*-deficient animals had an increased tumor incidence compared to control animals. This also resulted in a significant decrease in tumor-free survival (p-value = 0.001) as depicted in Figure 5.3B. As shown in h 5.1, 10 out of 11 (91%) *Fancf* $^{-/-}$ mice developed tumors compared to 6 out of 19 (32%) control animals. The tumor incidence was also more pronounced in females (p-value = 0.02, Supplementary Figure S5.1A) than in males (Supplementary Figure S5.1B, p-value = 0.08) although the number of mice was relatively low. We did not observe hematopoietic abnormalities indicative of anemia on blood smears taken during sacrifice.

Fancf $^{-/-}$ females develop granulosa cell tumors and luteoma of the ovary

Several different tumor types were observed, most notably lung tumors and ovarian tumors. Lung tumors developed in both *Fancf*-deficient and -proficient animals, whereas granulosa cell tumors (GCTs) were unique for the *Fancf* $^{-/-}$ mice occurring in 4 out of 7 female animals. One mouse even developed bilateral GCT and a fifth mouse a luteoma (see Table 5.1). Figure 5.3, panels C to F, shows low magnification pictures of the affected ovaries, higher magnifications of the respective tumors are shown in Figure 5.3G to 3J (10x) and Figure 5.3K to 3N (40X). One mouse showed, in addition to a GCT, epithelial morphological changes, including a tubular adenoma (Figure 5.3E). To our knowledge this is the first report in which FA pathway deficiency is correlated to an increased risk for granulosa cell tumors or luteoma of the ovary.

Fancf deficiency leads to hypogonadism and loss of primordial follicles

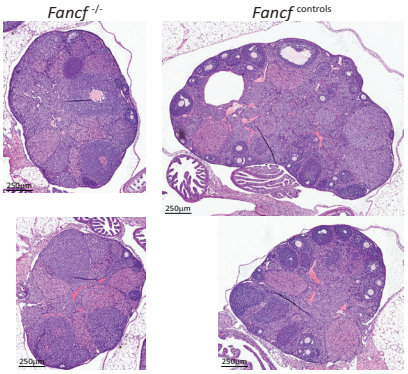
All FA mouse models described to date have gonads that function abnormally. A decrease in the number of primordial germ cells (PGC) has been described for FA mouse models [20]. In Fancc-deficient mice PGCs were lost due to a defect in proliferation

TABLE 5.1
Summary for the causes of death in the *Fancf* cohort.

Genotype	Gender	Days Lived	Tumor type
Fancf $^{-/-}$	Female	390	Granulosa cell tumor in ovarian and an invasive mammary carcinoma possibly initiated by hormone active ovarian tumor
Fancf $^{-/-}$	Female	432	Papillary lungcarcinoma
Fancf $^{-/-}$	Male	473	Papillary adenoma in Harderian gland
Fancf $^{-/-}$	Female	515	Granulosa cell tumors in both ovaries
Fancf $^{+/+}$	Female	593	Erythroleukemia
Fancf $^{-/-}$	Male	645	Papillary lungcarcinomas and hepatocellular carcinoma
Fancf $^{-/-}$	Female	661	Granulosa cell tumor and tubular adenoma in ovaries, lungcarcinomas with metastasis in liver and kidney
Fancf $^{-/-}$	Female	666	histiocytair B-cel lymphoma and hepatocellular carcinoma
Fancf $^{+/-}$	Male	669	Papillary lungcarcinoma
Fancf $^{+/+}$	Female	678	Papillary lungcarcinoma
Fancf $^{+/+}$	Male	700	Papillary cyst adenoma in Harderian gland and hepatocellular carcinoma
Fancf $^{-/-}$	Male	701	Papillary lungcarcinoma
Fancf $^{-/-}$	Female	704	Granulosa tumor in ovary and tumor in mammary gland.
Fancf $^{-/-}$	Female	711	Luteoma in ovary and leiomyoma in uterus, papillary lungcarcinoma
Fancf $^{+/+}$	Male	714	Hepatoma and papillary lungcarcinoma
Fancf $^{+/-}$	Female	732	Erythroleukaemia

and in subsequent survival [29]. We have therefore examined the gonads of the *Fancf*-deficient mice more carefully. Histological analysis of the testes from *Fancf*-deficient mice showed that the seminiferous tubules were devoid of germ cells and contained only Sertoli cells (Supplementary Figure S5.2). In addition, we sacrificed female mice of 7 and 14 weeks of age and performed extensive pathology and immunohistochemistry. We noted that the ovaries from *Fancf*-deficient animals were smaller and contained few growing follicles (Figure 5.4A). Ovaries from control animals contained several hundreds of primordial follicles and many growing follicles in both age groups (Figure 5.4B). In contrast, *Fancf* $^{-/-}$ animals showed a strong reduction in their primordial follicle pool. In the 7 weeks age group we observed a mixed phenotype with 2 *Fancf* $^{-/-}$ animals having comparable numbers of primordial follicles compared to control animals and 2 *Fancf* $^{-/-}$ animals having ovaries that were nearly completely depleted of primordial follicles (Figure 5.4B). At 14 weeks of age, all 3 *Fancf* $^{-/-}$ animals were almost or completely devoid of primordial follicles. We conclude that *Fancf* $^{-/-}$ animals displayed a rapid depletion of primordial follicles at a young age resulting in advanced ovarian aging.

A



B

Age	Genotype	Number of Fresh CL	Older generations CL present	Number primordial follicles
7 weeks	*Fancf* controls	5	yes	437
		7	yes	572
		0	yes	564
	Fancf -/-	0	no	0
		0	yes	377
		4	yes	446
		6	yes	4
14 weeks	*Fancf* controls	6	yes	482
		0	yes	284
		0	yes	498
		0	yes	319
	Fancf -/-	0	yes	0
		2	yes	3
		6	yes	1

CL= Corpus Luteum

FIGURE 5.4

(A) Cross-sections through the ovaries of *Fancf* $^{-/-}$ and control mice stained with hematoxylin and eosin low magnification (2.5x). (B) Table which summarizes the counted primordial follicles and corpus luteum (CL) present in the ovaries from *Fancf* $^{-/-}$ and control mice.

Remarkably, follicles could develop normally in the *Fancf*-deficient animals, despite the rapid depletion of primordial follicles, since we observed fresh corpora lutea in the *Fancf*-deficient ovaries, indicative of recent ovulation. During normal development most follicles degenerate during a process called atresia by apoptosis of the granulosa cells, unless rescued by FSH [30]. We assessed follicle development by performing a TUNEL assay and immunohistochemical staining for Caspase 3. We confirmed that in control ovaries follicle development was accompanied by apoptosis (Supplementary Figure S5.3). In the *Fancf*-deficient ovaries overall less apoptotic signal was noted as less developing follicles were present, but the scarce follicles that were maturing did show positive apoptotic staining as observed in control mice. This suggests that apoptosis of granulosa cells was not suppressed in *Fancf*-deficient animals (Supplementary Figure S5.3). Also Anti-Müllerian hormone staining (AMH), a marker for granulosa cells, was normal (data not shown).

It is striking to note that in both age groups *Fancf*-deficient animals contained corpus luteum (CL) despite showing a severe reduction of their primordial follicle pool (Figure 5.4B). This could indicate that CL in the *Fancf*-deficient animals persisted longer which could facilitate tumor development.

Fancf-deficient mice have an aberrant estrous cycle

We also assessed the estrous cycle in 2 months old *Fancf*-proficient and -deficient mice by cytologic evaluation of vaginal smears. The estrous cycle can be subdivided into four phases (proestrus, estrus, metestrus, and diestrus) and one cycle takes on average 4 days in the mouse. We saw that wild type mice cycled normally as all stages were present during our time course (Supplementary Table S5.1). In *Fancf*-deficient mice we saw that two animals cycled normally, while three animals did not progress

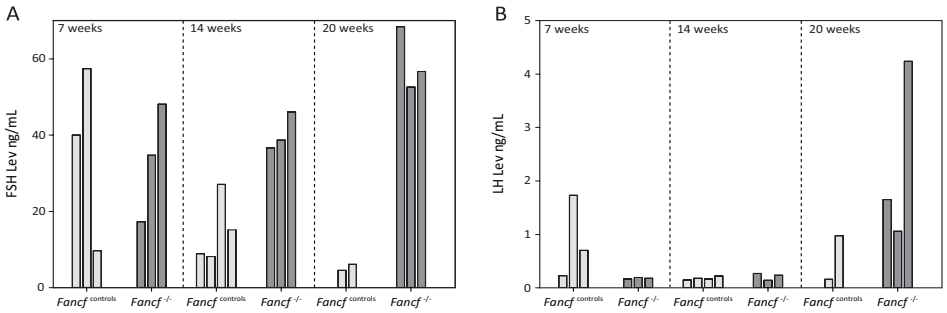


FIGURE 5.5

Gonadotropin levels (FSH (A) and LH (B)) in *Fancf*-deficient and proficient animals of 7, 14 and 20 weeks of levels in mouse serum. Gonadotropin levels were determined in 3 *Fancf*-proficient and 3 *Fancf* -deficient mice of 7 weeks of age, 4 *Fancf*-proficient and 3 *Fancf* -deficient mice of 14 weeks of age, and in 2 *Fancf*-proficient and 3 *Fancf* -deficient mice of 20 weeks of age.

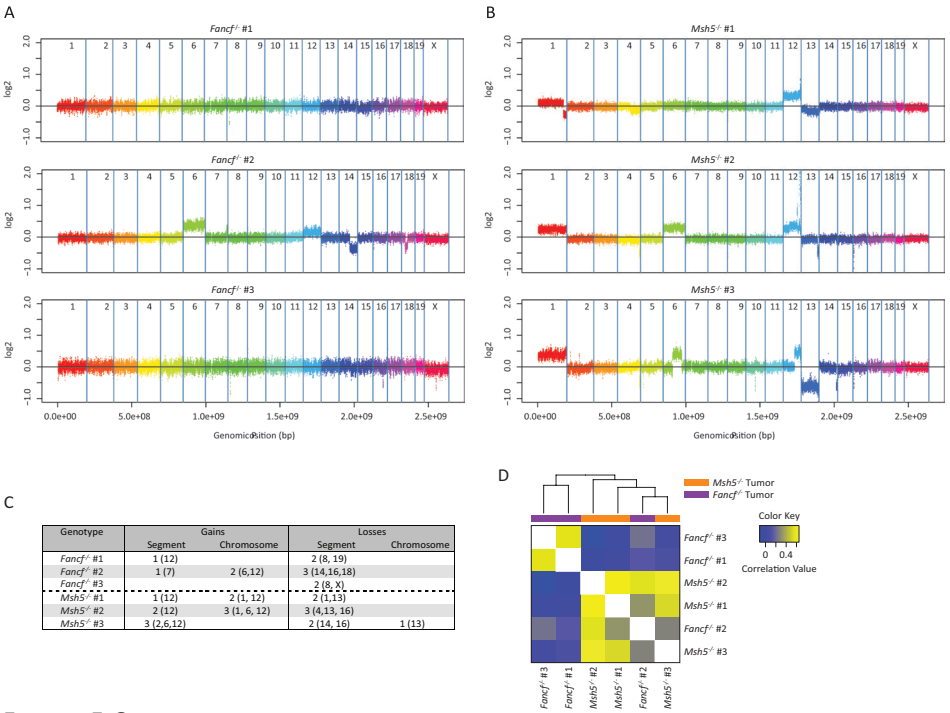
through different estrous phases. From two mice only few cells were visible which all resembled proestrus cytology, and one mouse showed only cells with diestrus cytological morphology.

Older *Fancf*-deficient mice have increased gonadotropin levels

To determine whether the hypogonadism and aberrant estrous cycling in *Fancf*-deficient mice also led to altered gonadotropin levels we collected serum samples from mice either 7, 14 or 20 weeks old. As shown in Figure 5.5A and 5B the 7 and 14 weeks old *Fancf*-proficient and -deficient mice had comparable FSH and LH levels but in the older cohort of 20 weeks, we observed an increased amount of FSH and LH in serum. The ovarian phenotype of the *Fancf*-deficient mice resembled that of women suffering from premature ovarian failure (POF) characterized by a lack of developing follicles and resulting in anestrus and hypergonadotrophism.

Fancf-deficient tumors show few copy number aberrations

We have shown that FANCF deficiency leads to genomic instability in MEFs and hypogonadism and hypergonadotrophism in mice. However, which of these two factors is causative for increased ovarian tumorigenesis in *Fancf*-deficient mice? To discriminate between these factors we made use of another mouse model that was created in our lab, the *Msh5* knockout mice. MSH5 promotes the synapsis of homologous chromosomes during meiosis and *Msh5* $^{-/-}$ mice lacked normal gonadal development and were infertile [31]. In addition, *Msh5* $^{-/-}$ mice developed granulosa cell tumors with a comparable latency as *Fancf* $^{-/-}$ mice (Supplementary Figure S5.4), although *Msh5* $^{-/-}$ cells had no overt mutator phenotype [31]. All these tumors arose in an identical mouse background (F1 between 129Ola and FVB). Pictures of these tumors are shown



C

Genotype	Gains		Losses	
	Segment	Chromosome	Segment	Chromosome
Fancf$^{-/-}$ #1	1 (12)		2 (8, 19)	
Fancf$^{-/-}$ #2	1 (7)	2 (6,12)	3 (14,16,18)	
Fancf$^{-/-}$ #3			2 (8, X)	
Msh5$^{-/-}$ #1	1 (12)	2 (1, 12)	2 (1,13)	
Msh5$^{-/-}$ #2	2 (12)	3 (1, 6, 12)	3 (4,13, 16)	
Msh5$^{-/-}$ #3	3 (2,6,12)		2 (14, 16)	1 (13)

FIGURE 5.6

Rainbow plots of high-resolution array comparative genomic hybridization (aCGH) profiles for 3 *Fancf*-deficient (A) and 3 *Msh5*-deficient GCTs (B). (C)Summary of copy number aberrations (CNA) found in aCGH profiles using segmentation analysis [26] . (D) Correlation matrix between the 3 *Fancf* and 3 *Msh5* aCGH profiles [27].

in Figure 5.3 for the FANCF model (tumor 1 in panels C, G, K, tumor 2 in panels D, H, L, and tumor 3 in panels F, J and N) and in supplemental Figure 5.4, panels B to G, for the MSH5 model. All tumors resembled each other on gross morphology although we noted a slightly higher mitotic index in tumor #1 and #3 of the *Fancf*$^{-/-}$ cohort.

To investigate whether *Fancf* deficiency leads to increased genomic instability, we measured DNA copy number aberrations (CNAs) in 3 *Fancf*-deficient and 3 *Msh5*-deficient granulosa cell tumors by high-resolution array comparative genomic hybridization (aCGH). Unfortunately, we could not include more tumors in our analysis since the remaining 2 tumors in our *Fancf* cohort were too small to isolate sufficient amount of DNA.

The *Msh5*-deficient GCTs (Figure 5.6B) showed a consistent pattern of CNAs. The CGH profile of one *Fancf*$^{-/-}$ tumor (#2) resembled those of *Msh5*-deficient tumors. The other *Fancf*-deficient tumors (#1 and #3) had a different CGH profile showing fewer genomic alterations. We summarized the CNAs in Figure 5.6C. To determine whether this gross observation represented a true correlation between the tumors, we performed unsupervised cluster analysis on the aCGH data. The cluster matrix in Fig-

ure 5.6D confirmed our observation as the 3 *Msh5* $^{-/-}$ tumors clustered together with *Fancf* $^{-/-}$ tumor #2 and the remaining 2 *Fancf* $^{-/-}$ tumors clustered together.

We conclude that in the *Msh5*-deficient model there is one set of genomic alterations that facilitated tumor development as 3 independent GCTs showed a remarkably similar CNA profile. However, in the *Fancf*-deficient tumors, 2 different types of GCTs developed: one that resembled the *Msh5* profile and one *Fancf* type that was characterized by fewer CNAs.

Discussion

Fancf-deficient mice resembled mice deficient for other FA core complex members: they showed hypogonadism and the cells had a disrupted FA pathway, as illustrated by MMC sensitivity [20,32]. We did not note perinatal lethality with or without a gender bias as has been observed for other FA core complex mice [20,23,32]. It is possible that this phenotype is not expressed in the 129Ola/FVB genetic background of our mice.

Increased ovarian tumor incidence for *Fancf*-deficient mice

Fancf-deficient mice showed a highly increased incidence of ovarian tumors (5 granulosa cell tumors and 1 luteoma in 7 animals). To our knowledge, this is the first report in which FA pathway deficiency is clearly correlated with an increased risk for this tumor type. Anecdotally, ovarian tumors have been reported in other FA mouse models: one Fanca-deficient mouse (129S6) developed a granulosa cell tumor at the age of 17 months in a cohort of 17 mice [33] and in a cohort of 54 Fancd2 mutant mice (129Sv-C57BL/6J background), 2 ovarian adenomas were observed [34]. Although the high incidence of ovarian tumors in the *Fancf* $^{-/-}$ mice is striking, these tumors may not unequivocally be attributed to the loss of FANCF function. Our *Fancf* cohort was an F1 between 129Ola and FVB and it is known that different mouse strains have different risks to develop ovarian tumors (see also additional discussion below). To claim a unique function for FANCF in suppression of ovarian cancer, we need to test the ovarian cancer incidence in other FA core complex models with an identical genetic background.

Ovarian tumorigenesis as a consequence of hypogonadism and hypergonadotropism

An intriguing question is whether the increased ovarian tumor incidence resulted from genomic instability due to the absence of FANCF, or was a secondary effect of hypogonadism. Several studies have suggested that the development of cysts and tumors can be a consequence of premature ovarian failure (POF) [35,36] formulated as the gonadotropin stimulation theory [37]. This theory states that during menopause or POF, the normal endocrine feedback loop is absent, and consequently serum levels of gonadotropins and proinflammatory cytokines rise and can stimulate ovarian

carcinogenesis. This correlation has also been observed in several mouse models. For example, Wv mice are infertile due to a depletion of primordial germ cells (PGCs) and these mice showed increased gonadotropin levels in serum and were prone to develop ovarian tubular adenomas [38]. Moreover, tumorigenesis was prevented by suppression of gonadotropin release [39]. Other mouse models demonstrated that high FSH and LH levels could also lead to an increased risk for ovarian tumors [40] and lowering gonadotropin levels resulted in reversal of the tumor phenotype [41]. The gonadal abnormalities in *Fancf*-deficient female mice are reminiscent of POF in humans [42], since we see hypogonadism, an aberrant estrus cycle, and hypergonadotropism.

Ovarian tumorigenesis as a consequence of FANCF deficiency

Together with the high incidence of GCTs in *Msh5*-deficient mice, the literature cited above supports the gonadotropin stimulation hypothesis of ovarian tumorigenesis. However, other studies have demonstrated a more complex etiology of ovarian tumor development. Several mouse models with gonadal abnormalities, for example Cdk2 null mice [43,44] or Foxo3a [45,46] null mice, did not develop ovarian tumors. Also, chemical destruction of follicles induced high gonadotropin levels, but this did not lead to development of ovarian tumors [47]. Data obtained from a mouse model showing chronic LH hypersecretion also illustrates a more complex relationship. Mice from the CF-1 background with elevated LH levels all developed ovarian tumors after 5 months. However, mice generated by crossing CF-1 with C57BL/6, SJL, or CD-1 strains did not develop ovarian tumors although the LH levels were comparably elevated as in the CF-1 mice [48]. These observations suggest that hypogonadism and hypergonadotrophism per se are not associated with an increased risk for ovarian tumors, but that unknown modifiers in different murine strains strongly influence the risk for ovarian cancer [49].

FANCF silencing has been observed in ovarian cancer, including GCT. This suggests that FA pathway inactivation through FANCF silencing could stimulate ovarian tumorigenesis. In addition, a recent report on the detailed analysis of ovarian carcinoma from the cancer genome atlas research network reported mutations in FA genes in 5.06% of tumors, suggestive for the involvement of a defective FA pathway in a subset of ovarian tumors [50]. To investigate whether tumors with a defective FA pathway follow a different aetiology, we have compared aCGH profiles of 3 *Fancf* $^{-/-}$ and 3 *Msh5* $^{-/-}$ GCTs. For the *Msh5* cohort we observed one distinct CNA profile in 3 independent tumors. We postulate that these tumors developed through a combination of abnormal gonadal function and modifiers specific to the 129Ola/FVB background. In our *Fancf* cohort with identical genetic background and similar abnormal gonadal function as the *Msh5* cohort, we found one tumor that resembled the *Msh5* CNA profile. However, two tumors had a different CGH profile which could point to a different aetiology of these tumors. We hypothesize that in these tumors a different type of genetic instability was present, which does not lead to gross chromosomal losses and gains, but rather to copy number neutral chromosomal translocations or small deletions and insertions, or nucleotide substitutions, that are not detected by CGH. In this respect, it is of note that FA cells showed an increased frequency of small dele-

tions at the HPRT locus compared to normal cells [51]. However, due to the small numbers of tumors available for aCGH analysis, this conclusion should be taken with caution. As in human 61% of GCTs have chromosomal aberrations [52], it is possible that we found the CNA low profile by chance only in the *Fancf* cohort and not in the *Msh5* cohort. Therefore, CGH analysis on a larger panel of *Fancf*-deficient and -proficient GCT tumors is needed to determine whether *Fancf* deficiency truly correlates with a low CNA-profile. Of the two types of GCT in humans, juvenile and adult, the latter is almost exclusively characterized by a somatic mutation in the FOXL2 transcription factor [53] and a low level of chromosomal instability [54]. It will be of interest to investigate whether a similar correlation exists in the mouse tumors.

In conclusion, we observed that *Fancf*-deficiency leads to FA pathway inactivation as illustrated by crosslinker sensitivity in MEFs. Moreover, *Fancf*-deficiency resulted in an increased risk for ovarian tumors. Our results provide an in vivo model for the co-occurrence of a POF-like phenotype in combination with FA pathway inactivation that is involved in the induction of ovarian tumorigenesis. Our results suggest that FANCF suppresses ovarian tumorigenesis through its role in normal gonadal development, but also by maintaining genome stability.

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Author Contribution

STB and HvV conceived and carried out experiments, JV, EDG, AvWm MB, MvV, AO and PK carried out experiments, FA and MvdV analysed data. STB wrote the paper and STB, HvV, JdW and HtR were involved in the final writing of the paper and had final approval of the submitted and published versions.

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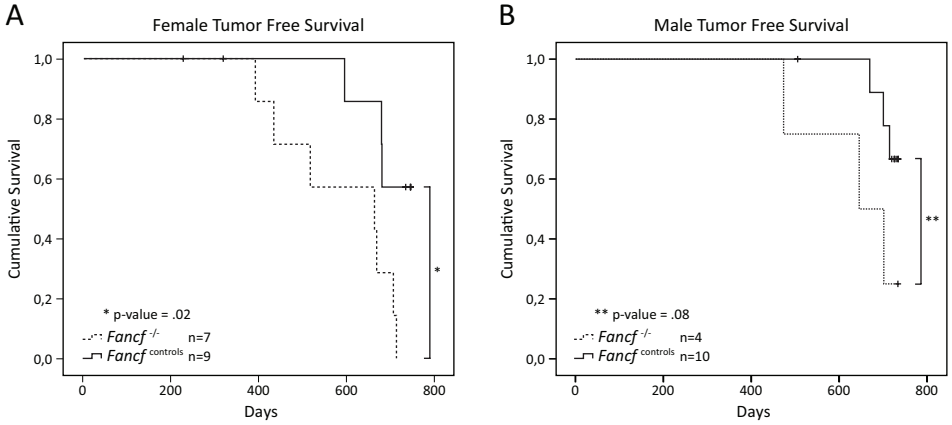
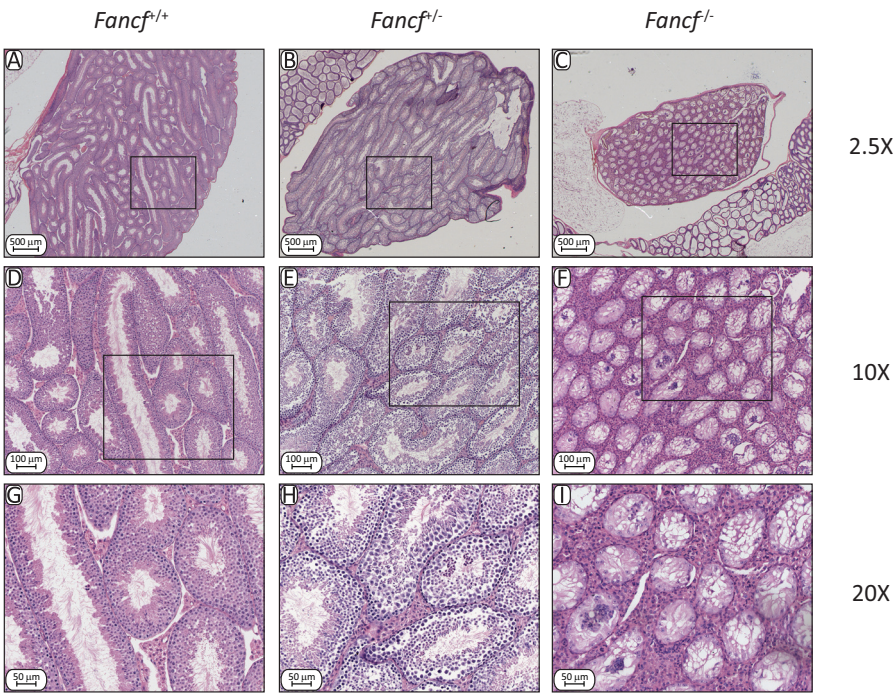
Supplemental Figures



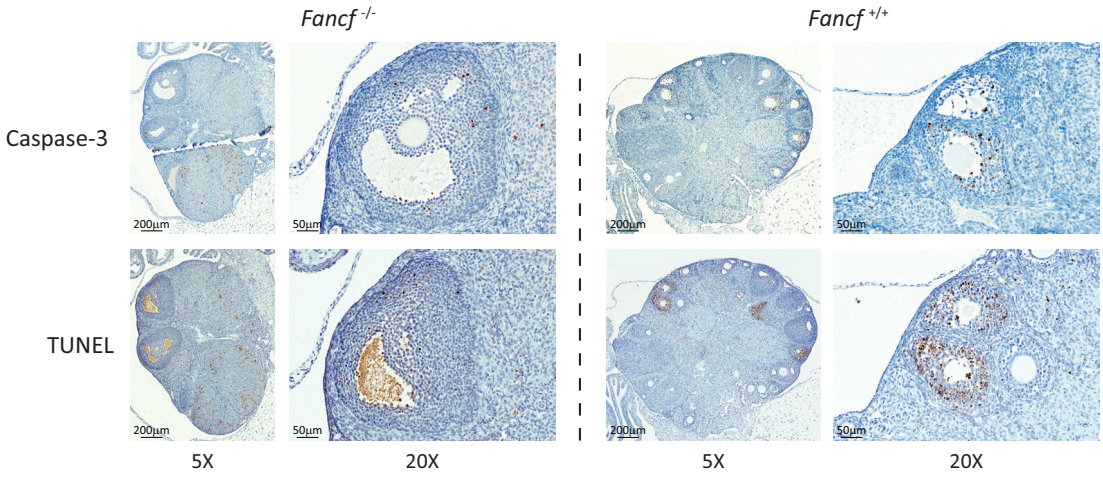
SUPPLEMENTARY FIGURE S5.1

Kaplan Meier survival curves of tumor free survival for *Fancf*$^{-/-}$ mice and control littermates stratified for gender (A) females, (B) males.



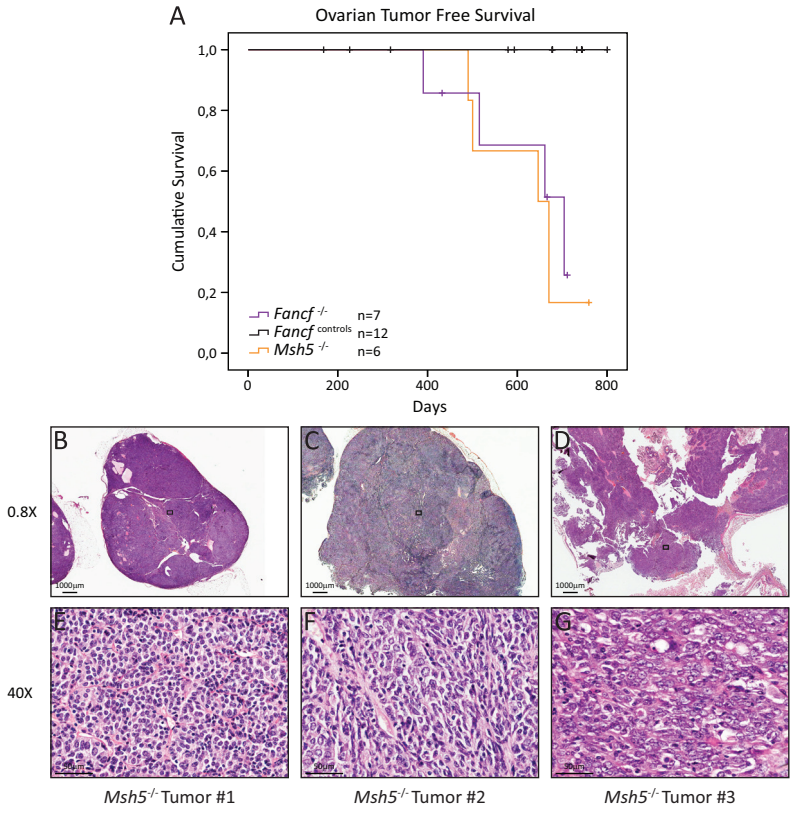
SUPPLEMENTARY FIGURE S5.2

Histological analysis of testis of 6 months old *Fancf*$^{-/-}$ mice and controls. (A-C) cross-sections through the testes stained with hematoxylin and eosin of a wild-type (A), heterozygous (B) or homozygous mouse (C) at low magnification (4x). D-F: 10X magnification of the squared regions depicted in A-C that contain several seminiferous tubules. G-I: 20X magnification of the squared regions depicted in D-F.



Supplementary Figure S5.3

Ovaries from 2 months old *Fancf*-deficient and proficient animals were harvested and stained for the apoptotic markers TUNEL and caspase 3. Normal follicle development is accompanied by positive apoptotic staining as can be seen in the *Fancf*-wild type ovaries (bottom row), in the *Fancf*-deficient animals overall apoptotic staining is reduced but still present in some follicles and corpora lutea (upper row).



Supplementary Figure S5.4

Kaplan Meier curves of ovarian tumor free survival for *Fancf*$^{-/-}$, *Msh5*$^{-/-}$ and control mice. Panels B to G show 3 *Msh5*-deficient granulosa cell tumors with different magnifications on which aCGH was performed (#1, #2 and #3).

Supplementary Table S5.1

Fancf deficient mice have an aberrant estrus cycle Vaginal swabs were obtained from 2 months old mice daily for 5 consecutive days on 10 am and were subdivided into the four phases of the estrus cycle; proestrus (P), estrus (E), metestrus (M), and diestrus (D).

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Fancf $^{-/-}$	P	E	M	D	D
Fancf $^{-/-}$	D	P	P	E	M
Fancf $^{-/-}$	P	P	P	P	P
Fancf $^{-/-}$	P	P	P	P	P
Fancf $^{-/-}$	D	D	D	D	D
Fancf $^{+/+}$	E	M	D	D	D
Fancf $^{+/+}$	D	D	P	E	D
Fancf $^{+/-}$	M	D	D	P	P
Fancf $^{+/+}$	M	D	D	P	E

Chapter 6

FANCM functions independently of the FA pathway as a tumor suppressor in the intestine

Manuscript in preparation

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Abstract

Fanconi anemia (FA) is a recessive disorder caused by mutations in one of at least 15 genes and characterized by anemia and cancer predisposition[1]. The identification of FA genes has uncovered a cellular pathway involved in the protection against DNA interstrand crosslinking (ICL) agents, the FA pathway[2]. The contribution of a compromised FA pathway to chromosomal instability and tumorigenesis has been studied by gene disruptions in mice. These mouse models recapitulated cellular characteristics of FA, but failed to show the pronounced cancer proneness of FA patients[3,4].

We crossed *Fancf*- or *Fancm*-deficient mice into the *Apc*^{Min} mouse model to investigate whether a compromised FA pathway accelerates tumorigenesis in a sensitized background. We found that FANCM deficiency strongly accelerated intestinal tumor development in the *Apc*^{Min} model, whereas FANCF deficiency had no effect. These data support a role for FANCM as a tumor suppressor independent of the FA pathway.

Introduction

Increased tumor incidence has been reported in *Fancc*, *Fancd2*, *Fancf* or *Fancm* mouse models, but occurred relatively late[3,4]. It has also been demonstrated that *Fancc*- or *Fancd2*- deficiency decreased the latency of tumor development in p53 mutant mice[5,6]. However, in all these mouse models it remained unclear whether accelerated tumorigenesis was due to increased genomic instability.

Here, we have investigated whether genomic instability associated with FA pathway deficiency can modulate tumor development in a model for intestinal tumorigenesis. To this end we have crossed *Fancf*- and *Fancm*-deficient mice into the *Apc*$^{Min/+}$ model.

Both, *Fancf*- and *Fancm*-deficient mice demonstrated a cellular FA phenotype with ICL hypersensitivity, absent (*Fancf*) or reduced (*Fancm*) FANCD2 monoubiquitination, and a comparable increase in chromosomal breaks after ICL treatment[7,8].

The *Apc*$^{Min/+}$ mouse model carries a truncating mutation at codon 850 of the adenomatous polyposis coli (*Apc*) gene and suffers from multiple intestinal neoplasia (Min)[9,10]. *Apc*$^{Min/+}$ mice develop adenomas after loss of the wild-type APC allele[11]. The frequency of tumorigenesis was increased in mice which were either chromosomal or microsatellite unstable (CIN or MIN), reflecting that loss of the wild-type *Apc* allele could occur through chromosomal instability or mutations. For example, increased tumorigenesis occurred in *Msh2*-deficient mice through somatic mutations in the wild-type *Apc* allele whereas *Blm* mutant mice rapidly developed adenomas through increased mitotic recombination leading to loss of heterozygosity (LOH)[12-14]. We speculate that since FA cells have an increased frequency of small deletions at the HPRT locus and are characterized by chromosomal instability, a disrupted FA pathway could modulate tumorigenesis in the *Apc*^{Min} background[15,16].

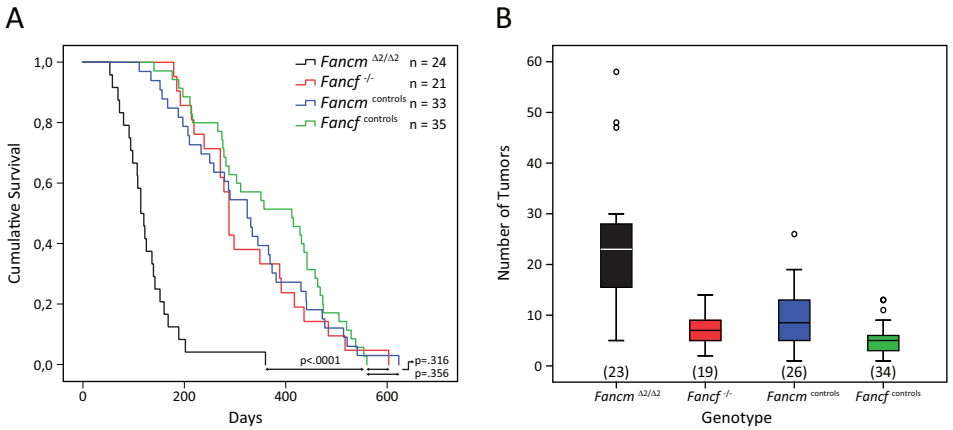


FIGURE 6.1

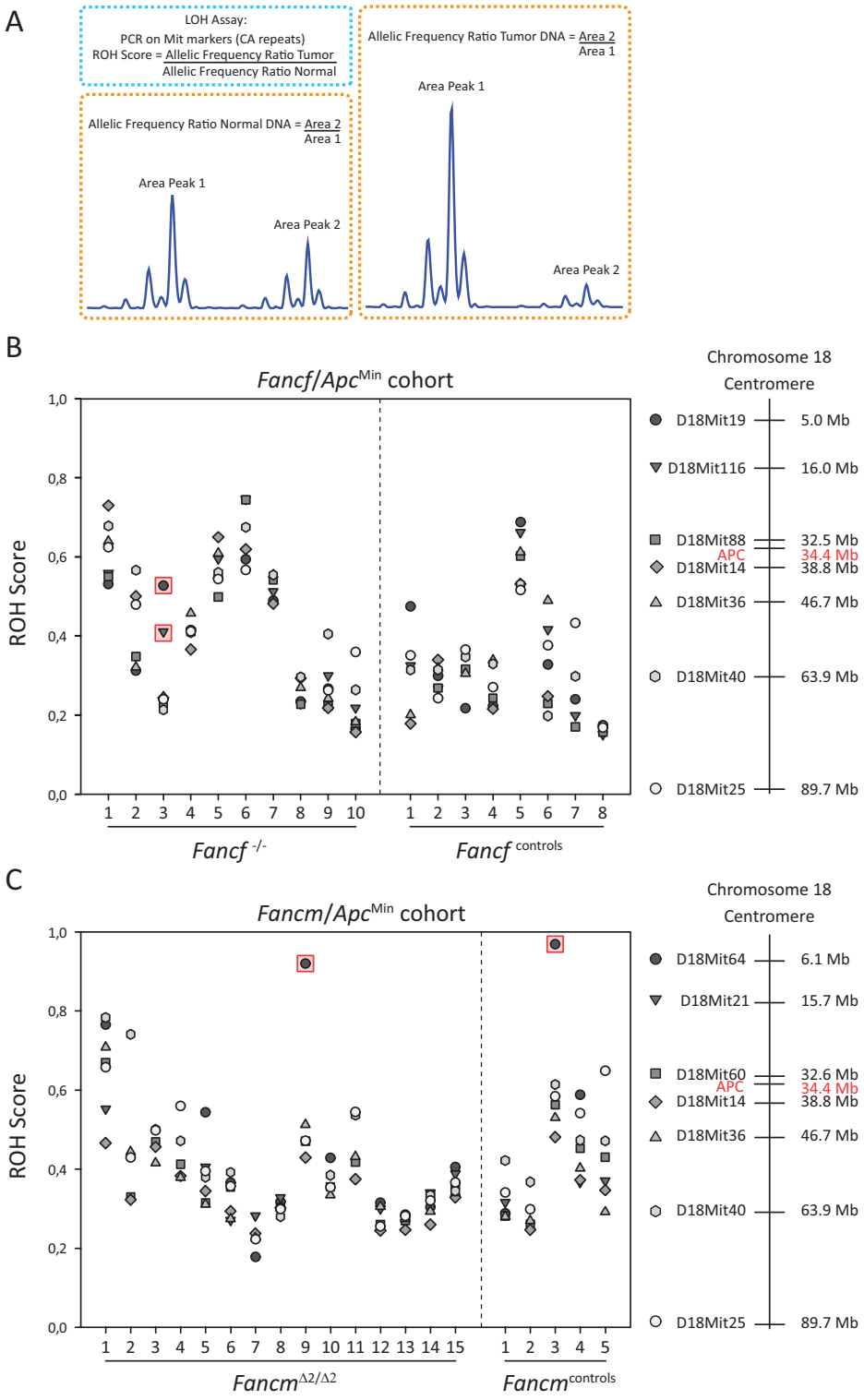
Reduced survival and increased tumor formation in *Fancm*-deficient *Apc*^{Min} mice. (A) Kaplan Meier survival curves of *Fancm*- and *Fancf*-proficient and deficient *Apc*$^{Min/+}$ mice. (B) Boxplots of the number of tumors counted throughout the intestine. The number of tumors in *Fancm*$^{-/-}$ mice was the only significant outlier (p-value <0.05) as calculated by Bonferroni comparison (SPSS version 17).

Results

We generated *Fancf* and *Fancm* deficient and proficient *Apc*^{Min} mice and followed these mice for tumor development. As genetic background strongly influences tumor susceptibility[17], we excluded the homozygous presence of the sensitive allele of modifier of min 1 (*Mom1*^{S}). FA pathway proficient *Apc*^{Min} mice succumbed to intestinal tumors at a mean age of 400 days (Figure 6.1A). Surprisingly, *Fancf* deficiency did not accelerate tumorigenesis in the *Apc*^{Min} model, whereas *Fancm* deficiency did (Figure 6.1A), although both proteins act in the FA pathway.

Since we did not observe differences between wild-type and heterozygous animals we grouped both genotypes together as controls (Supplementary Figure S6.1A and S6.1B)[7,8]. Also the number of adenomas was significantly increased in *Fancm*-deficient mice compared to *Fancf*-deficient mice and littermate controls (Figure 6.1B). We observed no difference in the location of tumor development between the cohorts, most adenomas developing in the small intestine and only a minority in the colon[9]. It has been demonstrated that *Apc*^{Min} mice are also predisposed to mammary tumors[18]. In accordance, some mice that were sacrificed due to intestinal tumors had also developed tumors in the mammary gland (2 in *Fancf*$^{-/-}$, 6 in *Fancf*^{controls}, 2 in *Fancm*$^{\Delta 2/\Delta 2}$, and 2 in *Fancm*^{controls}). *Fancm*-deficient mice developed these tumors again at a younger age compared to the control cohorts, whereas *Fancf*-deficiency did not accelerate tumorigenesis in this tissue type.

We next investigated by which mechanism the wild-type *Apc* allele was lost in *Fancm*-deficient tumors[11]. FANCM is involved in the suppression of sister chromatid ex-


A
LOH Assay:
PCR on Mit markers (CA repeats)
ROH Score = Allelic Frequency Ratio Tumor / Allelic Frequency Ratio Normal
Allelic Frequency Ratio Normal DNA = Area 2 / Area 1
Area Peak 1
Area Peak 2
Allelic Frequency Ratio Tumor DNA = Area 2 / Area 1
Area Peak 1
Area Peak 2
B
Fancf/Apc^Min cohort
ROH Score
1,0
0,8
0,6
0,4
0,2
0,0
1 2 3 4 5 6 7 8 9 10
Fancf -/-
1 2 3 4 5 6 7 8
Fancf controls
Chromosome 18
Centromere
D18Mit19 5.0 Mb
D18Mit116 16.0 Mb
D18Mit88 32.5 Mb
APC 34.4 Mb
D18Mit14 38.8 Mb
D18Mit36 46.7 Mb
D18Mit40 63.9 Mb
D18Mit25 89.7 Mb
C
Fancm/Apc^Min cohort
ROH Score
1,0
0,8
0,6
0,4
0,2
0,0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Fancm^Δ2/Δ2
1 2 3 4 5
Fancm^controls
Chromosome 18
Centromere
D18Mit64 6.1 Mb
D18Mit21 15.7 Mb
D18Mit60 32.6 Mb
APC 34.4 Mb
D18Mit14 38.8 Mb
D18Mit36 46.7 Mb
D18Mit40 63.9 Mb
D18Mit25 89.7 Mb

changes (SCEs)[7,19,20] and can recruit the BLM helicase to stalled replication forks[19]. We speculated that *Fancm* deficiency could also promote non-sister chromatid exchanges (nSCEs) as has been observed in the *Blm* mutant mouse model[13]. To test this hypothesis we performed LOH analysis using seven polymorphic microsatellite markers along chromosome 18, the chromosome that contains the *Apc* gene. LOH of all polymorphic markers would be indicative for loss of the wild-type *Apc* allele by chromosomal homozygosis or a single mitotic recombination event near the centromere[21]. If increased mitotic recombination around the *Apc* locus had occurred a mosaic pattern of LOH and retention of heterozygosity (ROH) would be detected[13]. We plotted ROH scores on the *y*-axis for each Mit marker and calculated p-values to determine significant outliers (see Figure 6.2). Most adenomas had undergone LOH of all seven markers indicative of loss of the wild-type *Apc* allele by either chromosomal homozygosis or mitotic recombination near the centromere which we can not distinguish. One tumor in the *Fancf* cohort (#3) showed ROH of two adjacent Mitmarkers proximal to the *Apc* gene (D18Mit19 and D18Mit116) indicating a mitotic recombination event between D18Mit116 and D18Mit88. One *Fancm*-deficient tumor (#9) and one *Fancm*-control tumor (#3) showed ROH of D18Mit64, indicative of a mitotic recombination event between D18Mit64 and D18Mit21. These data show that mitotic recombination was not increased in *Fancm*-deficient tumors.

The absence of increased crossover frequency was confirmed by whole exome sequencing of tumor and spleen DNA from one *Fancm*-deficient mouse (tumor #3). We reasoned that if FANCM deficiency promoted crossover frequency, this would not be restricted to chromosome 18 but apparent throughout the genome. We confirmed that this tumor had developed with a stable karyotype as known for Apc^{Min} tumors (data not shown)[21]. We calculated the b-allele frequency (BAF) and plotted the difference between the BAF^{tumor} minus the BAF^{spleen} (ΔBAF) for all chromosomes. As can be appreciated from Supplementary Figure S6.2A only chromosome 18 showed a significant difference in ΔBAF and we confirmed that this tumor had undergone LOH of all informative SNPs along chromosome 18 (Supplementary Figure S6.2B). Therefore, the increased tumorigenesis in the *Fancm* mouse model was not associated with an increased nSCE incidence, but it remained possible that *Fancm* deficiency promoted single mitotic recombination events near the centromere.

FIGURE 6.2
(opposite page)
Mechanism of LOH in *Fancf* and *Fancm* Apc^{Min} cohorts (A) Schematic outline for the determination of ROH scores by using Mitmarker analysis. Seven polymorphic markers along chromosome 18 were used to asses the incidence of LOH. (B) ROH score of $Fancf^{-/-}$ Apc^{Min} and control Apc^{Min} tumors. (C) ROH scores of $Fancm^{-/-}$ Apc^{Min} and control Apc^{Min} tumors. Most tumors had lost the wild-type *Apc* allele through either chromosomal homozygosis or mitotic recombination near the centromere; 4 Mitmarkers in 3 tumors ($Fancf^{-/-}$ #3, $Fancm^{\Delta 2/\Delta 2}$ #9 and *Fancm* control #3) showed a significant deviation and were considered outliers indicative for retention of heterozygosity (Mitmarker D18Mit19 and D18Mit116 corrected p-value <0.0001, D18Mit64 corrected p-value 0.035 for both tumors).

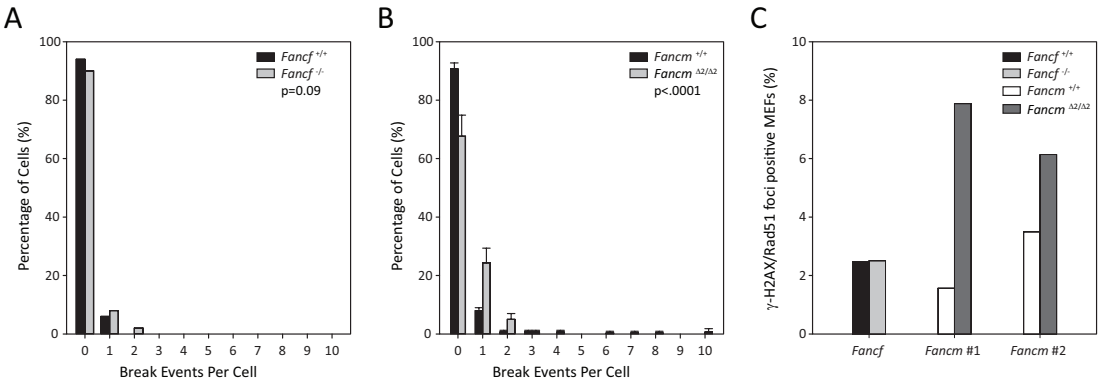


FIGURE 6.3

Increased genomic instability in *Fancm*-deficient MEFs. Chromosomal breakage analysis for *Fancf*$^{-/-}$ (A) and *Fancm*$^{\Delta 2/\Delta 2}$ (B) primary MEF cultures with littermate controls (C) Nuclear foci from one primary *Fancf*$^{-/-}$ MEF culture with littermate control and two *Fancm*$^{\Delta 2/\Delta 2}$ primary MEF cultures with littermate controls were scored for the presence of γH2AX/Rad51 foci (>5 foci per cell). 100 cells were scored per experiment.

Alternatively, FANCM deficiency may contribute to tumorigenesis by loss of the intra-S-phase checkpoint. RNAi-mediated knockdown of FANCM in human cells abrogated the ATR/CHK1 mediated intra-S-phase checkpoint after replication stress[22]. However, we did not find abrogation of this checkpoint in *Fancm*-deficient immortalized MEFs, early passage primary MEFs, and splenocytes (Supplementary Figure S6.3). This is in agreement with data obtained in other model organisms[23] and could be explained by differences in mechanisms of FANCM deficiency (FANCM depletion versus disruption) and differences in checkpoint activation between species[24].

6

To investigate the role of FANCM in maintaining genome stability we assessed the presence of DNA damage in primary early passage MEFs. First we scored the number of chromosomal breaks in metaphase spreads. Whereas control MEFs or *Fancf*-deficient MEFs exhibited few break events, *Fancm*-deficient MEFs showed a significant increase in the number of break events per cell (compare Figure 6.3A and 6.3B). We also assessed the presence of DSBs by immunofluorescence microscopy of nuclear foci positive for both γ-H2AX and Rad51[25]. As shown in Figure 6.3C, cells with more than 5 foci were more often present in two different primary *Fancm*-deficient MEF cultures than in *Fancf*-deficient or control cultures. An increase in γ-H2AX foci has also been observed in HeLa and DT40 cells deficient for FANCM[22,26]. These data demonstrate that *Fancm* deficiency caused genomic instability in the absence of exogenous genotoxic stress whereas *Fancf*-deficient MEFs showed increased chromosomal breakage only after ICL treatment[8].

Discussion

A role for FANCM independent of the FA pathway has been postulated in the stabilization of stalled replication forks to ensure normal replication fork progression[7,26,27]. It has been postulated that stabilization of a stalled replication fork involves fork reversal: the (re)annealing of the template and nascent strands initiated by RAD51 mediated recombination results in a chickenfoot or Holliday junction (HJ) structure that prevents the collapse of stalled replication forks[24,28,29]. We suggest that FANCM plays a role in both fork reversal and reinstallation of the replication fork and in the absence of FANCM stalled replication forks can not be efficiently reinstalled (Supplementary Figure S6.4). This is supported by the ability of FANCM to remodel replication forks through its branchpoint translocation activity (BPT) *in vitro*[30-32] and the requirement for FANCM for efficient replication fork progression *in vivo*[26,27].

A stalled replication fork can be reinstalled through RAD51-mediated invasion of the leading strand into the reannealed template strands leading to the formation of a double Holliday junction or hemicatenane[24]. This double Holliday junction can be dissolved by the BTR complex (BLM helicase, topoisomerase IIIα, RMI1 and RMI2) to reinstall the replication fork[33]. We envisage that FANCM could promote this reaction through its BPT activity[30-32], facilitating RAD51 dependent invasion and through subsequent recruitment of the BTR complex[19]. An alternative to remodelling of the stalled replication fork would be the approach of a replication fork from the opposite orientation stimulated by dormant origin firing. In agreement, in the absence of FANCM increased dormant origin firing has been observed after replication fork stalling and this ensured completion of replication[26]. Interestingly, this increased origin firing was not observed in other FA mutant cells[26].

However, the availability of dormant origins is limited in certain regions of the genome such as in common fragile sites[34]. Therefore, when FANCM mediated replication fork remodelling is inhibited and origin firing is limited, the chicken foot structure may be targeted by Holliday junction resolvases[33]. This would result in collapsed replication forks and the formation of DSBs (see Supplementary Figure S6.4)[24]. The repair of DSBs by the canonical HDR machinery can lead to increased crossovers and LOH when the non-sister chromatid is used as template. Although not proven directly, we envisage this scenario would take place predominantly near centromeres. Thus, the acceleration of mitotic recombination events near the centromeric tip of chromosome 18 provides a mechanism for the increased tumor susceptibility in *Fancm*-deficient $Apc^{Min/+}$ mice. FANCM-mediated fork reversal and reinstallation can occur in the context of ICL recognition and repair but, also upon other forms of replication stress. Apparently, ICLs were a minor cause for fork reversal in mice as a defect in ICL repair, i.e. *Fancf* deficiency did not accelerate tumorigenesis in the Apc^{Min} mice.

Methods

Generation of mice

Fancf- and *Fancm*- deficient mice were described previously[7,8]. *Apc* $^{Min/+}$ were originally created in an ethylnitrosourea (ENU) treated mutagenesis screen in the C57/BL/6J (BL6) background[9]. These mice were first backcrossed (>10 generations) into the Balb/c background and subsequently backcrossed (>10 generations) into the FVB background. Chromosome 18 was therefore polymorphic for BL6, Balb/c and FVB whereas the remaining genome contained predominantly FVB sequences. FVB *Fancf* $^{+/-}$ mice were intercrossed with *Apc*$^{Min/+}$ mice to obtain *Fancf* $^{+/-}$;*Apc*$^{Min/+}$ and these mice were then crossed with 129Ola *Fancf* $^{+/-}$ mice to generate an F1 of FVB/Ola129 *Fancf* $^{-/-}$, *Fancf* $^{+/-}$, *Fancf* $^{+/+}$ mice mutant for *Apc* . FVB *Fancm* $^{+/\Delta 2}$ mice were intercrossed with *Apc* $^{Min/+}$ mice to obtain *Fancm*$^{+/\Delta 2}$ *Apc* $^{Min/+}$ and these mice were then crossed with F3 backcrossed BL6 *Fancm*$^{+/\Delta 2}$ mice to generate a semi F1 of FVB/BL6 *Fancm*$^{\Delta 2/\Delta 2}$, *Fancm*$^{+/\Delta 2}$, *Fancm*$^{+/+}$ mice mutant for *Apc*.

Animal husbandry

Mice were housed in conventional cages, with a standard maintenance diet and water provided *ad libitum*. All animal experiments were approved by the local ethical review committee on animal experimentation and conducted according to Dutch legislation. Animals were examined on a regular basis for tumor development and sacrificed when tumors were present or when animals were otherwise unhealthy. Surviving animals were sacrificed at 2 years of age. Overall and tumor-free survival curves were generated using SPSS software (Version 17), statistical significance between genotypes was determined using built-in analysis for survival curves consisting of a log rank test yielding a p-value.

Histological analysis

Mice were killed and isolated organs were fixed in 4% formaldehyde in PBS. Fixed organs were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Large tumors were frozen at -80 °C for DNA analysis.

Intestinal tumor load

The small and large intestines were fixed as flat spirals and embedded in paraffin blocks for sectioning. Intestinal tumor load was assessed macroscopically and by examining H&E-stained longitudinal sections. In addition, slides were scanned using a Scanscope XT digital slide scanner (Aperio, Vista, CA, USA) and tumor numbers were determined by analyzing with Imagescope software (Aperio).

Loss of heterozygosity analysis

PCR amplification of polymorphic microsatellite sequences on chromosome 18 was

performed using 1 µL of DNA prepared from approximately 10 to 20 mg of tissue dissected from single intestinal adenomas and spleen controls. Fluorescently labeled PCR products were analyzed using peak area ratios to determine loss and retention of heterozygosity in tumor tissue. Seven different microsatellite markers polymorphic between the genetic strains were used (D18Mit19, D18Mit116, D18Mit88, D18Mit14, D18Mit36, D18Mit40, and D18Mit25 for *Fancf* cohort; D18Mit64, D18Mit121, D18Mit60, D18Mit14, D18Mit36, D18Mit40, and D18Mit25 for *Fancm* cohort). Primers and fluorescently labeled primers were obtained from Sigma-Aldrich Company Ltd. ROH ratio scores represent the marker intensity ratio from tumor DNA divided by the marker intensity ratio from normal DNA. To find significant outliers we calculated a p-value for single or double outliers using the Grubbs test for outliers[35]. We only considered outliers that had a positive deviance from the sample mean for outlier testing as these reflect Mit markers that had a comparable wild-type to mutant allele ratio in both tumor and spleen. We corrected the resulting p-values for multiple testing using the Benjamini-Hochberg approach[36].

Chromosomal Breakage Analysis

For each primary MEF culture, 50 metaphases were analyzed for chromatid-type chromosomal abnormalities. To quantify chromosomal abnormalities the interchange aberrations were converted into break events. All scoring was performed blindly to eliminate counting bias. We calculated p-values through a χ^2 test as implemented in Microsoft Excel (2003).

Immunofluorescence

Cells were cultured on cover slides, washed with PBS, and fixed for 5 min using 4% paraformaldehyde (Merck). Cells were permeabilized by 0.1% Triton X-100 (Sigma) in PBS for 5 min. Subsequently, cells were washed three times using staining buffer (0.15% glycine [Merck], 0.5% bovine serum albumine [BSA; Sigma] in PBS), and were incubated for 60 min at room temperature in staining buffer. Cells were incubated for 4 h and 1 h with primary and secondary antibodies, respectively. Bleaching was prevented by Vectashield (Vector Laboratories). The primary antibodies used were rabbit polyclonal Rad51 (a gift from Dr. R. Kanaar) and mouse monoclonal phosphorylated H2AX (Upstate Biotechnologies) in 1:2500 and 1:100 dilutions in staining buffer, respectively. Secondary antibodies used were Alexa 488-labeled chicken anti-mouse and Alexa 568-labeled goat anti-rabbit antibodies (Molecular Probes), and these were used in a 1:200 dilution in staining buffer. DNA was stained using To-Pro3 dye (Molecular Probes).

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Supplementary Materials and Methods

Whole exome sequencing and LOH analysis

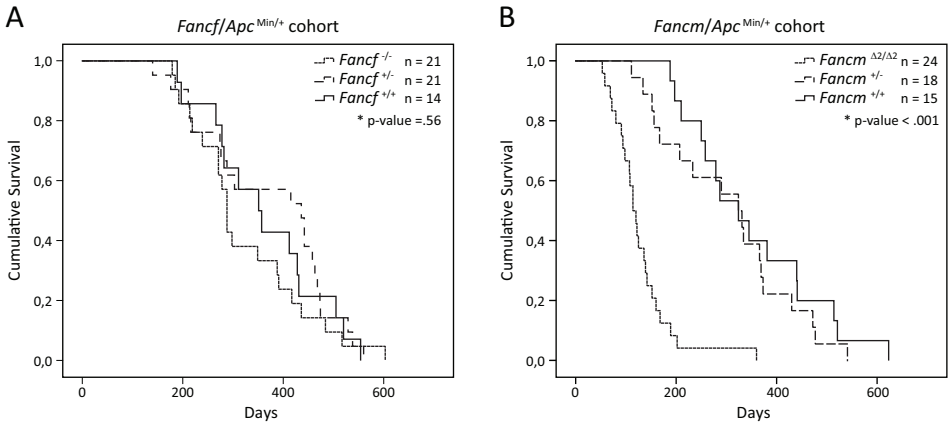
DNA was PCR-amplified for 18 cycles using 2 units of Phusion polymerase. PCR clean-up was performed using AMPure beads (Agencourt BioSciences Corporation Beverly, MA, USA) following the manufacturer's protocol. We used the Agilent SureSelect XT Mouse Whole Exome Kit for capture according to the manufacturer's specifications. We prepared Genome Analyzer paired-end flow cells on the supplied Illumina cluster station and generated 76-bp paired-end sequence reads on the Illumina Genome Analyzer platform following the manufacturer's protocol. Images from the Genome Analyzer were processed using the manufacturer's software to generate FASTQ sequence files. Paired end sequence reads were aligned to the mouse reference genome NCBI build 37 (C57/BL/6J) using BWA[37]. PCR duplicated reads and reads with a mapping quality below 60 were filtered out. The remaining reads were used to call variants using Samtools[38].

For the LOH analysis we first considered all polymorphic positions in the exome of the control (spleen) sample, and for each of the positions, the B-allele count is the number of reads with non-reference- (B-allele) at that position. For a polymorphic position i, let N_i be the total number of reads mapped to that position (i.e. depth-of-coverage); thus the B-allele count B_i follows a binomial distribution Binomial (p_i, N_i). A binomial that rejects the null hypothesis: $p_i = 0.5$ can be used to detect LOH at each polymorphic position. Segmentation is done using CBS algorithm based on the absolute difference in B-allele frequencies $|BAF^{tumor} - BAF^{spleen}|$

Immunoblots and antibodies

Cells were treated for the indicated period and harvested through scrapping and subsequently lysed for 30 min on ice in ELB buffer (150 mM NaCl, 50 mM Hepes at pH 7.5, 5 mM EDTA, 0.1% NP-40) containing protease inhibitors (Complete; Roche). Protein concentrations were measured using the BCA protein assay kit (Pierce). Equal protein concentrations were loaded on a 4–12% Tris-Acetate NuPAGE gradient gel (Invitrogen) and proteins were separated by gel-electrophoresis, according to the manufacturer's protocol. The primary antibodies used were mouse monoclonal Cdc25A (F6-sc7389; Santa Cruz Biotechnology, gift from Andrew Deans), mouse monoclonal Chk1 (G4-sc8408; Santa Cruz Biotechnology), rabbit monoclonal phospho-Chk1 Ser345 (Cell Signaling), goat polyclonal CDK4 (C22-sc-260; Santa Cruz Biotechnology). Secondary antibodies used were HRP-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit, and HRP-conjugated rabbit anti-goat (DakoCytomation).

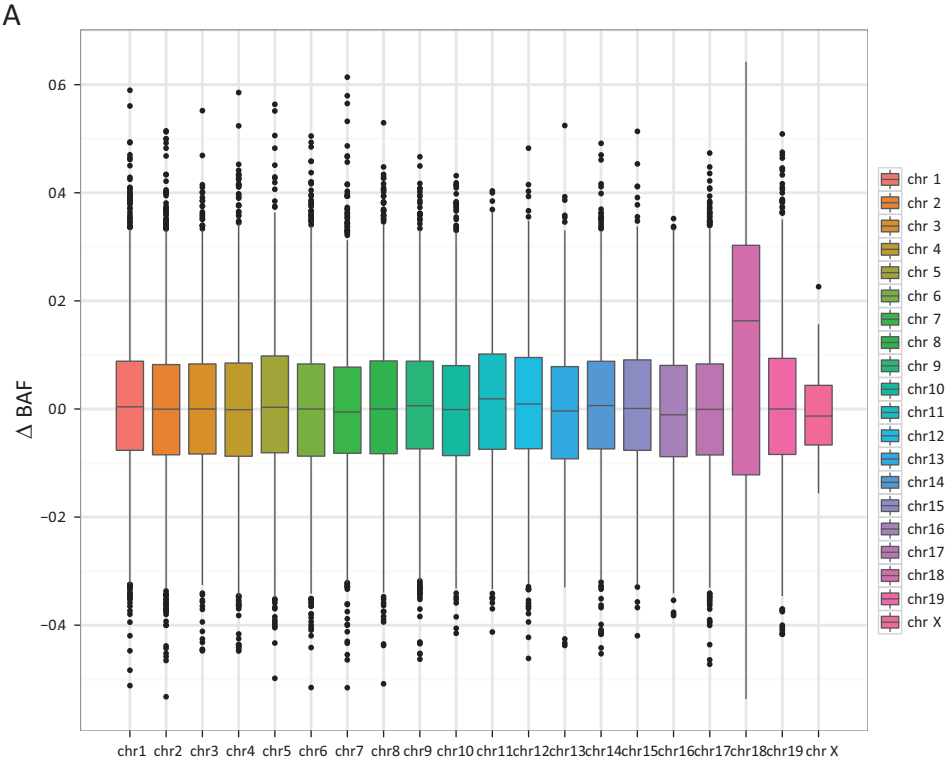
Supplementary Figures



SUPPLEMENTARY FIGURE S6.1

Kaplan-Meier curves subdivided per genotype (A) *Fancf*/*Apc*^{Min} cohort, (B) *Fancm*/*Apc*^{Min} cohort. P-values were calculated in SPSS with a log-rank test comparing heterozygous and homozygous animals.

A


Δ BAF
06
04
02
00
−02
−04
chr1 chr2 chr3 chr4 chr5 chr6 chr7 chr8 chr9 chr10 chr11 chr12 chr13 chr14 chr15 chr16 chr17 chr18 chr19 chr X
chr 1
chr 2
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chr 4
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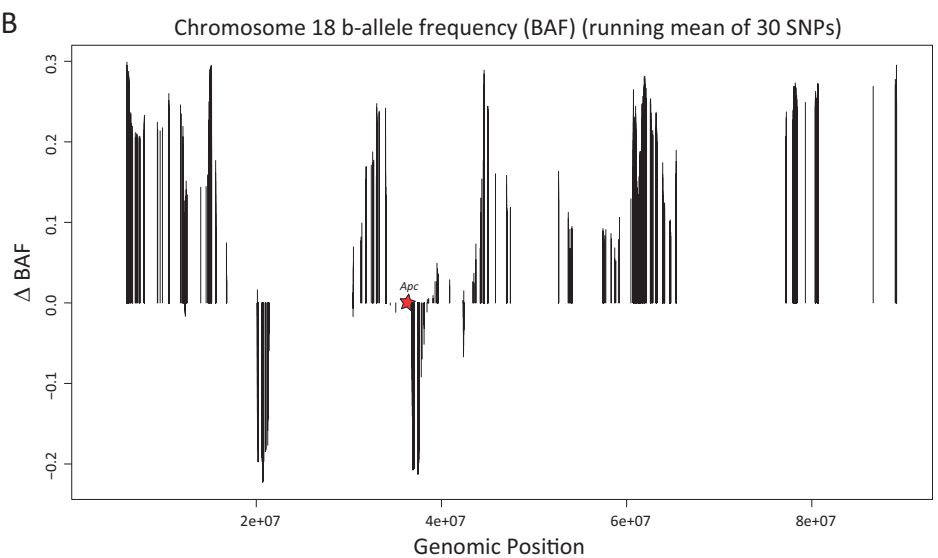
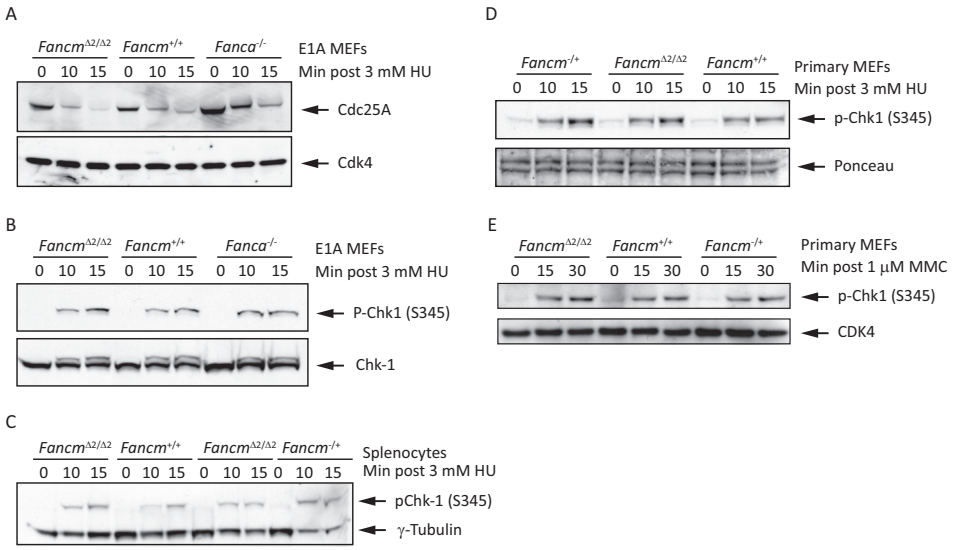
B


Chromosome 18 b-allele frequency (BAF) (running mean of 30 SNPs)
Δ BAF
0.3
0.2
0.1
0.0
−0.1
−0.2
Apc
2e+07
4e+07
6e+07
8e+07
Genomic Position

Supplementary Figure S6.2

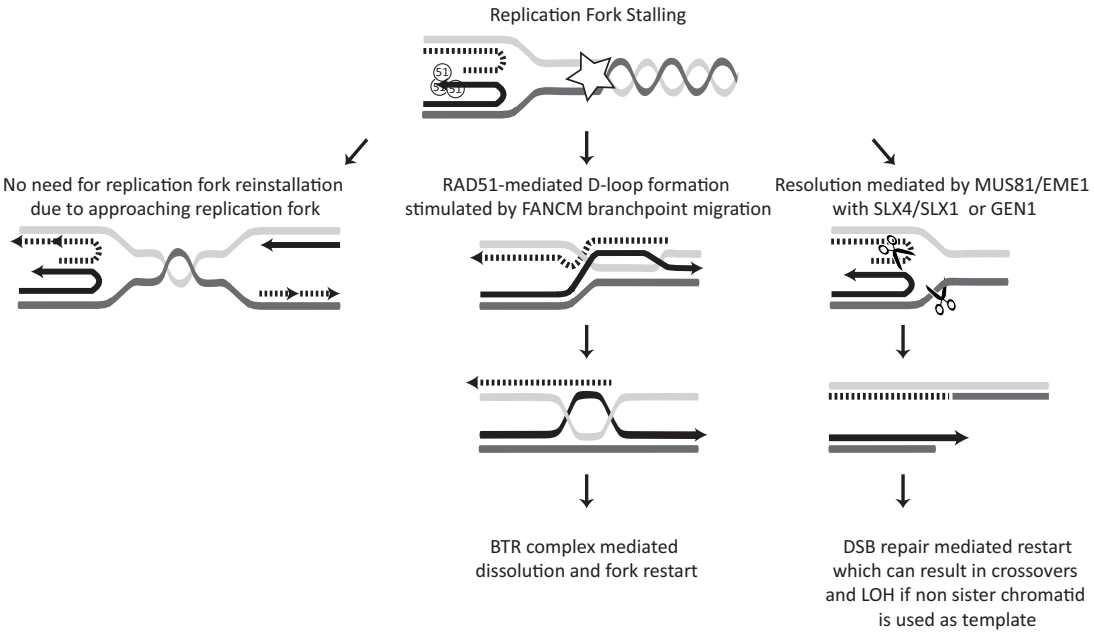
(opposite page)

(A) Boxplots of the ΔBAF (BAF^{tumor} – BAF^{spleen}) are plotted per chromosome. Only chromosome 18 showed a significant deviation of ΔBAF whereas the rest of the genome showed no regions that had undergone LOH. (B) Zoom in of the ΔBAF distribution along chromosome 18. The *Apc*^{Min} mutation was originally made in the C57/BL/6J genetic background[9] (the reference or A-allele) and subsequently backcrossed to Balb/c and FVB (the non-reference genome or B-allele). Therefore, the *Fancm*$^{-/-}$ *Apc*^{Min} mouse contains a mosaic chromosome 18 where the mutant *Apc* allele is predominantly FVB with part Balb/c (both B-allele) and a small region surrounding the *Apc*^{Min} mutation which is still descendent from the C57/BL/6J genetic background (A-allele). As can be appreciated from this ΔBAF plot of chromosome 18 the majority of the SNPs had become the B-allele (mutant allele). Of exception are two peaks representing predominantly A-alleles, these peaks represent regions in the mutant *Apc* allele that sill contained C57/BL/6J. No areas were found indicative of ROH (ΔBAF = 0). Hence, loss of the wild-type *Apc* allele was due to LOH either by chromosomal homozygosis or mitotic recombination near the centromere, where no informative SNPs were found with whole exome sequencing.



Supplementary Figure S6.3

E1A immortalized MEFs of the indicated genotypes do not show an inactive intra-S-phase checkpoint stimulated by hydroxyurea (HU) treatment as normal Cdc25-A (A) degradation and Chk1 poshporylation (B) is observed. Primary splenocyts do not show a defective intra-S-phase checkpoint (C). Primary MEF cultures of early passage and indicated genotypes show a normal intra-S-phase checkpoint after HU treatment (D) or MMC treatment (E).



Supplementary Figure S6.4
Model for genomic instability in the absence of FANCM.

Chapter 7

Discussion

Fanconi anemia - the FA pathway for ICL repair

FA cells are characterized by a hypersensitivity to ICL agents, which is manifested by increased chromosomal breakage, a prolonged G2 arrest, and reduced survival. Based on this shared phenotype, all FA proteins are thought to function together in the so-called FA pathway of ICL repair. ICL agents induce either intra,- or interstrand crosslinks in the DNA of which the interstrand crosslink is considered the most cytotoxic, since it prevents strand segregation thereby blocking replication, transcription, and chromatin maintenance [1]. ICLs therefore need to be removed and the integrity of the DNA restored in order for a cell to survive. ICL repair requires the coordination of multiple different repair pathways, which varies per cell cycle phase. The FA pathway is thought to play a critical role in the coordination of these different repair pathways and some FA proteins are also directly involved in the repair process itself.

Experiments in cell free *Xenopus laevis* egg extracts have uncovered several molecular details of replication-dependent repair of a plasmid containing an ICL and pointed to a central role for the FA pathway [2,3]. In this system ICL repair was mediated by two replication forks which converged on the ICL and subsequently stalled leading strand synthesis initially 20 nucleotides (cisplatin) or 24 nucleotides (nitrogen mustard-like) from the lesion [2]. Next, one of the two leading strands approached the ICL up to 1 nucleotide and subsequent repair took place through ICL unhooking, translesion synthesis (TLS) and double strand break (DSB) repair through homologous recombination (HR) [2-4]. The *Xenopus* egg extract plasmid based ICL repair system is extremely useful to investigate the contribution of different proteins in repair and to identify different repair intermediates formed during repair. However, the approach does have some limitations. The occurrence of the initial pausing and the need for two replication forks to converge on the ICL could be dependent on the type of ICL used as, for example, a triplex-forming-oligonucleotide (TFO)-psoralen ICL did not result in the stalling of two replication forks at -20 nt of the ICL [5]. Moreover, the repair of a plasmid ICL could differ from the repair of a chromosomal ICL especially with respect to the likelihood of two replication forks approaching an ICL from opposite directions.

A substantial number of models have postulated the formation of a DSB as an obligate intermediate for efficient ICL repair in the late S and G2 phases of the cell cycle [1-4,6]. This DSB is formed as a consequence of ICL unhooking in close vicinity of the stalled replication fork and is subsequently repaired through the canonical DSB repair machinery involving RAD51-mediated HR [1-4,6]. However, a DSB is a highly toxic lesion and we speculate that, if possible, a cell will aim to prevent such a lesion. Here, we postulate an alternative model for recombination-dependent but DSB-independent ICL repair (Figure 1). This ICL repair model uses the tools a G1 cell has to its disposal for ICL repair: nucleotide excision repair (NER) and TLS [7,8], but now exploited in the context of DNA replication. The efficiency of ICL repair is greatly increased in the presence of replication [2,3,9]. This is likely due to the requirement for replication fork stalling as a mechanism for ICL recognition and to induce an efficient cellular response [10]. In our alternative model for recombination-dependent

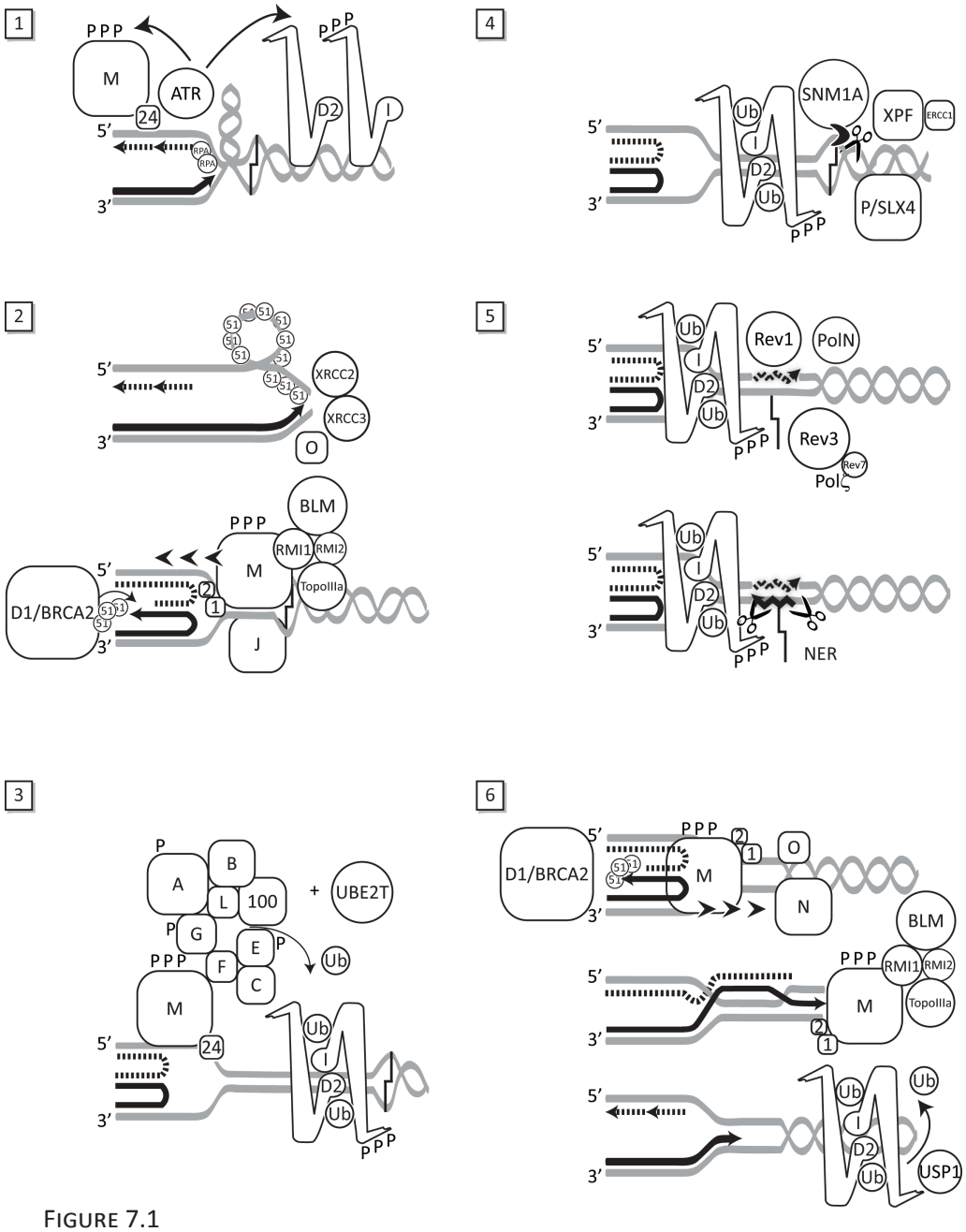


FIGURE 7.1

A model for recombination-dependent but DSB-independent ICL repair orchestrated by the FA pathway.

(1) Stalling of a replication fork approaching an interstrand crosslink

(2) Initiation of fork reversal by RAD51-mediated HR and formation of a chicken foot structure

(3) Recruitment of the FA core complex by FANCM and monoubiquitination of the ID complex

(4) ID-stimulated unhooking of the ICL and exonucleolytic degradation

(5) Translesion synthesis across the unhooked lesion followed by NER of the unhooked lesion

(6) Reinstallation of the replication fork involving RAD51-mediated invasion of the leading strand into the repaired double-stranded template mediated by FANCM and followed by BTR complex dependent dissolution of the double Holliday intermediate. Deubiquitination of the IC complex by USP1 marks the completion of ICL repair.

but DSB independent ICL repair the FA pathway plays a central role. An active FA pathway is critical for ICL repair primarily in the context of replication as repair efficiency is severely reduced in its absence [2,3,9]. In addition, we postulate that FANCM can play a role in replication fork remodeling both within and outside the context of ICL repair, i.e., independent of the FA pathway.

ICL repair - step 1, Helix distortion and replication stalling

An ICL prevents strand segregation and causes topological stress in front of an approaching replication fork, which subsequently leads to stalling of this replication fork. It is possible that this situation already activates the FA pathway. The aberrant replication structure could be recognized by FAAP24, which recognizes ssDNA, and results in recruitment of FANCM through the C-terminus of FANCM [11]. Both proteins are enriched on chromatin in a co-dependent manner [12] and FANCM's chromatin enrichment is also dependent on the interaction with MHF1 and MHF2 which bind dsDNA [13,14]. In addition, FANCM is phosphorylated after ICL exposure or after HU treatment and this modification is thought to increase chromatin affinity [12]. FANCD2 and FANCI are also recruited to the chromatin after replication fork stalling and this relies on the ATR-mediated phosphorylation of FANCI [15].

ICL repair - step 2, Stabilization of stalled replication fork through chicken foot structure

Proteins involved in HR are essential for ICL repair as mutants defective for HR are sensitive to ICL agents [16]. The FA and HR pathways are intertwined since RAD51 and FANCD2 are both recruited to nuclear foci after ICL damage and the two pathways are epistatic for ICL repair [17,18]. However, we argue that the substrate for HR is not a DSB formed after ICL unhooking but rather the stalled replication fork itself. The recombination product is a reversed replication fork, a so-called chicken foot structure. Homologous recombination is mediated by RAD51 molecules which assemble into a protein filament that wraps around ssDNA and induces pairing with, and strand invasion into an intact double-stranded homologous DNA molecule to initiate recombination [19]. *In vitro* DNA strand exchange assays have demonstrated that the Escherichia coli homolog of RAD51, RecA, could load on circular single-stranded DNA without the necessity for a free DNA end and mediate pairing with a complementary strand present in a linear duplex DNA molecule [20]. A similar reaction may occur at a stalled replication fork in which a RAD51 filament can form around the single-stranded template for lagging strand synthesis. The filament may subsequently promote invasion of the homologous DNA duplex that had been formed by leading strand synthesis thus catalyzing the reannealing of the original template strands.

Further regression of the stalled replication fork requires a helicase. This process may involve the human helicase-like transcription factor (HLTF) by removing proteins from the stalled replication fork [21], but could also be stimulated by the helicase FANCJ which interacts with the BLM helicase and stimulates its unwinding of a dam-

aged DNA substrate [27]. The displaced leading and lagging strands may now anneal to form the chicken foot structure with a classical Holliday junction.

Recent data have added evidence for recombination dependent fork reversal prior to ICL repair in mammalian cells. Firstly, it was determined that RAD51 is involved in DNA replication, independent of its role in DSB repair [22]. Secondly, RAD51 depletion decreased replication restart after hydroxyurea (HU) treatment, a drug that stalls replication forks [23]. Thirdly, it was shown that RAD51 during plasmid-ICL repair in *Xenopus* egg extracts was loaded onto stalled replication forks prior to DSB formation confirming a DNA replication function for RAD51 independent of homology-directed repair (HDR) of DSBs [4]. And fourthly, it was demonstrated that FANCD1/BRCA2 is involved in the protection of stalled replication forks from nucleolytic degradation by MRE11 independent from HDR [24]. FANCD1/BRCA2 mediated fork protection was dependent on the C-terminus of FANCD1/BRCA2 which stabilizes RAD51 filaments independent of BRC repeat mediated RAD51 interaction and this C-terminus-RAD51 interaction was dispensable for HDR of DSBs [24]. Moreover, the stabilization of stalled forks rather than promoting DSB repair was important for RAD51's role in maintaining genomic integrity after fork stalling [24]. FANCO/RAD51C could contribute to efficient fork reversal by regulating the nuclear translocation of RAD51 and by promoting checkpoint signaling to delay cell cycle progression in response to DNA damage [25,26].

Therefore, we postulate that HR proteins, such as RAD51, XRCC2, XRCC3, but also the FA proteins FANCD1/BRCA2, FANCO/RAD51C and potentially FANCJ, participate in ICL repair by generating and stabilizing a chicken foot structure. It is important to note that during the process of fork reversal FANCD1/BRCA2 is only needed to stabilize RAD51 on the nascent leading DNA strand to prevent degradation of the replication fork [24]. We hypothesize that fork reversal by HR proteins is independent of FANCD2 and FANCI monoubiquitination since normal RAD51 foci form in patient cells with FA core complex defects and FA-D2, but not FA-D1 mutant cells [27]. Moreover, depletion of FANCD2 resulted in normal RAD51 recruitment in DNA replication dependent ICL repair and vice versa [4].

It is possible, that FANCM plays a role in this fork reversal step, although we also speculate on a role for FANCM later in ICL repair (see step 6). FANCM contains several conserved domains: a DEAH helicase domain in its N-terminus and a degenerate endonuclease domain in its C-terminus [28,29]. Although FANCM has no helicase activity [28,30], purified FANCM could bind to Holliday junctions and replication forks and promote branchpoint translocation (BPT) [30,31]. These activities were ATP dependent and FAAP24 independent [30,31]. Purified FANCM was also able to dissociate D-loops and promote the reversal of replication forks *in vitro*. [30-32]. In addition, FANCM is conserved in *Saccharomyces cerevisiae* (Mph1) and *Schizosaccharomyces pombe* (Fml1), and these orthologs were also capable of replication fork regression and branch migration of a chicken foot structure [33,34]. As FANCM was shown to have bi-directional BPT activity it is possible that FANCM helps to stabilize the reversed fork by translocating the branchpoint of the chicken foot further away from the ICL

[30-32]. Moreover, it was shown that the replication fork regression capacity of FANCM was increased by interaction with MHF1 and MHF2 [13,14].

This FANCM-dependent remodeling may also involve recruitment of the BTR complex. This complex consists of the BLM helicase, topoisomerase IIIα, RMI1 and RMI2 and promotes the dissolution of double Holliday junctions [35]. FANCM contains a conserved protein-protein interaction domain, MM2, which interacts with RMI1 and topoisomerase IIIα and recruits the BLM helicase to stalled replication forks [36]. It has also been shown that BLM can regress stalled replication forks *in vitro* [37]. Therefore, we propose that FANCM can recruit the BTR complex to move the regressed fork away from the ICL by branchpoint translocation and make the lesion accessible for repair.

ICL repair - step 3, ID monoubiquitination by the FA core complex

The FA core complex is enriched on the chromatin through the interaction with FANCM via FANCF, which is mediated by MM1, a conserved protein-protein interaction motif on FANCM [36]. Eight of the FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) interact in a multi-subunit complex called the FA core complex which also contains four FA-associated proteins (FAAP24, FAAP100, MHF1, and MHF2) [13,38]. The FA core complex acts as an E3-ubiquitin ligase and monoubiquitinates FANCI and FANCD2, also called the ID complex [39]. FANCL contains a conserved PHD/RING finger domain necessary for the E3-ligase activity [40] and UBE2T acts as the E2-ubiquitin ligase for this reaction [41]. However, all subunits of the FA core complex are necessary for monoubiquitination of the ID complex. In addition, several members of the FA core complex are phosphorylated [42]. The FA core complex is constitutively assembled and stable throughout the cell cycle, but the enrichment of the FA core complex, UBE2T, and FANCD2 to chromatin occurs independently [43,44]. FANCM is part of the FA core complex [28] but not essential for its assembly [12,45] and FANCD2 monoubiquitination is observed in FANCM-deficient human or murine cells albeit at strongly reduced levels [45,46]. In addition, ATP-hydrolysis by FANCM is dispensable for monoubiquitination of FANCD2 but necessary for ICL resistance [31].

Recently, the structure of the ID complex has been solved, revealing that it folds into a saxophone-like structure with the monoubiquitinated lysine residues embedded in the interface [47]. Monoubiquitination stabilizes the ID complex on the chromatin [3]. The ID structure also suggested that the ID complex has two sets of dsDNA/ssDNA binding sites, which could bind structures generated by the replication fork stalled at an ICL. In agreement with the stalled replication fork as the substrate for ID and FA core complex recruitment is the potent monoubiquitination of the ID complex not only after ICL exposure but also after HU treatment.

ICL repair - step 4, ICL unhooking

The monoubiquitination of FANCD2 and FANCI by the FA core complex has been

shown to be necessary for efficient incision of the DNA backbone flanking the ICL, the so-called unhooking step [3,4]. In our recombination-dependent but DSB independent model for ICL repair we envision that the removal of the ICL occurs analogous to NER-dependent ICL repair observed in G1 [7,8]. Additional molecular details of the unhooking steps in ICL repair and the role of the monoubiquitinated ID complex have recently become more clear with the discovery of Fanconi anemia Associated Nuclease 1 (FAN1) [48-51] and FANCP/SLX4 [52,53].

FAN1 is a nuclease with both 3'-5' exonuclease and 5'-flap endonuclease activity and contains a ubiquitin binding zinc finger (UBZ) domain at its N terminus. FAN1 is thought to interact with monoubiquitinated FANCD2 through its UBZ domain. It is therefore tempting to speculate that monoubiquitinated FANCD2 recruits FAN1 to the site of the ICL to initiate the first unhooking step in ICL repair, especially since the ID complex is required for efficient incision of an ICL [3]. However, homozygous deletion of the FAN1 locus did not cause FA [54] and disruption of Fan1 in DT40 cells did not cause FA like chromatid breaks [55]. These data make it unclear whether FAN1 acts in ICL repair and, if so, at which step.

FANCP/SLX4 is a protein mutated in FA. Cells from patients of the FA-P complementation group show normal FANCD2 monoubiquitination, indicative of normal FA core complex function, but are ICL sensitive [52,53]. FANCP/SLX4 acts as a scaffolding protein through its multiple protein interaction domains including two UBZ domains at its N-terminus [56-58]. In agreement with this scaffolding function, FANCP/SLX4 interacts with endonucleases (SLX1, XPF-ERCC1, MUS81-EME1), components of the shelterin telomere protection complex (TRF2-RAP1), the mismatch repair complex (MSH2-MSH3) and other proteins [56-58]. The interaction of FANCP/SLX4 with XPF-ERCC1 and its ability to enhance nuclease activity towards replication forks and 3' flap structures appears to be important for ICL repair [57,58]. Therefore, FANCP/SLX4, potentially in conjunction with XPF/ERCC1 could facilitate the unhooking event necessary for ICL repair [59]. Another recently identified candidate that could play a crucial role in ICL repair is SNM1A. This 5'–3' exonuclease could load from a single DNA nick and degrade DNA past an ICL [60]. Moreover, SNM1A depleted cells were sensitive to ICLs [60].

ICL repair - step 5, Translesion bypass and nucleotide excision repair mediated ICL removal

After the first unhooking of the ICL and possible degradation, the DNA strand must be extended past the lesion and replication needs to be resumed. However, normal replicative DNA polymerases are not capable to replicate ICL-damaged DNA and therefore translesion synthesis (TLS) polymerases are required. Experiments in DT40 cells and mice have indicated that REV1 and REV3 could be the TLS polymerases active in ICL repair since mutants are sensitive to ICLs and this sensitivity is epistatic with FANCC [16,18]. Additionally, experiments in the *Xenopus* egg system have indicated that REV1 inserts one nucleotide across the cross-linked base which depends

on monoubiquitinated FANCD2 [3]. This strand is then further extended by REV3 (catalytic subunit of POLζ) [2,3].

Subsequently, the damaged DNA containing the ICL needs to be removed. This step in the repair process could be mediated by nucleases and polymerases which are also active in NER and is likely also mediated by nucleases interacting with FANCP/SLX4. Also exonucleases could be involved in removal of damaged DNA. A possible candidate is the nuclease MRE11 [61] that was shown to be recruited to ICLs in an FA dependent manner [62] and to be important for ICL resistance [63].

Both unhooking of the ICL (step 4) and TLS past the ICL (step 5) are inhibited in the absence of monoubiquitinated FANCD2/FANCI, which highlights the importance of the FA pathway for ICL repair [3].

ICL repair - step 6, Replication restart through reinstallation of the replication fork

After ICL repair, a replication fork coming from the opposite direction may finish replication. However, when this is not possible, the chicken foot structure needs to be dismantled to allow replication restart. How this occurs is unclear but one model may be the following [64]. As a chicken foot structure is analogous to a Holliday junction it could be processed identically. Branch migration of the Holliday junction towards the original site of replication fork stalling, is catalyzed by FANCM/BRT until the lagging strand has completely reannealed to its template. Indeed, FANCM was found to promote branch migration, stimulated by MHF1 and MHF2, which is necessary for replication fork restart [13,14]. Complete reannealing of the leading strand again requires RAD51-dependent HR. One possibility is that the 3'-end of the leading strand, which is covered by RAD51 filament (step 2), invades the double-stranded template strand to form a double Holliday junction or hemicatenane. A different FA protein, FANCN/PALB2, could aid in the formation of the D-loop necessary for fork restart. FANCN/PALB2 is the interaction partner of FANCD1/BRCA2 and was demonstrated to have a strong preference for binding D-loop structures and stimulated RAD51-catalyzed D-loop formation through its interaction with FANCD1/BRCA2 to promote strand invasion [65,66].

Normally, a double Holliday junction is dissolved by the BTR complex [67,68]. Dissolution involves the approach of two branchpoints to ultimately form a catenated structure that can be dismantled by topoisomerase activity. This would directly lead to reinstallation of the replication fork. We envisage that in the absence of FANCM, reinstallation of the replication fork is blocked. In this case the chicken foot structure may be targeted by Holliday junction resolvases, which would lead to collapsed replication forks with double strand breaks. Subsequent HDR-directed DSB repair may lead to reinstallation of the replication fork, but in this case this may be accompanied by SCEs .There is substantial evidence for the occurrence of a recombination and dissolution based mechanism for replication fork restart. Firstly, in *Xenopus* egg extracts

hemicatenanes were shown to be intermediates in ICL repair [4]. In addition, it was demonstrated that dissolution is the preferred mechanism over resolution to remodel double Holliday junctions [67]. This function of FANCM explains the increased incidence of spontaneously occurring DSBs and SCEs in *Fancm*-deficient cells.

Unique role for FANCM independent of the FA pathway

We propose that FANCM has a critical role in replication fork metabolism which extends beyond its role in the FA pathway, and is critical for maintaining genomic stability. We speculate that FANCM prevents the occurrence of DSBs by its ability to promote efficient replication fork remodeling. It is possible that in the absence of FANCM, stalled replication forks cannot effectively regress which may lead to cleavage of the stalled fork and the accumulation of DSBs [69,70]. However, we favor a model in which in the absence of FANCM, reinstallation of the replication fork cannot. This could lead to resolves targeting the stalled replication fork for resolution resulting in SCEs and DSBs [36,67]. We conclude this on the basis of several experimental observations outlined in the thesis and published by others.

Firstly, we found that *FANCM*-deficient human cells are uniquely sensitive to camptothecin (CPT) and UV [chapter 2]. CPT blocks replication and could stimulate fork reversal at low concentrations. The inability of *FANCM*-deficient cells to respond adequately to replication blocking lesions could explain the observed sensitivity to CPT and UV, which cause fork stalling. CPT sensitivity was also observed in FANCM-depleted HeLa cells or *FANCM* mutant DT40 cells and correlated with decreased replication fork progression, a phenotype that was not observed in other FA mutant cells [69,70]. FANCM has also been shown to mediate normal checkpoint response independent of the FA pathway [71]. The inability to stabilize stalled replication forks could result in an inadequate DNA damage response. We did however not observe exquisite camptothecin sensitivity or checkpoint defects in our murine *Fancm*-deficient cells [chapter 6]. This can be explained by differences in the sensing of stalled replication forks in rodent and human cells [64] or by differences between transformed cells and primary MEFs. Discrepancies between different FANCM-deficient cell types have been noted as FANCM-depleted HeLa cells showed resistance to HU and aphidicolin (APH) whereas DT40 cells were sensitive to these agents [69,70].

Secondly, we demonstrated that *Fancm*-deficient MEFs had a significant increase in spontaneous Sister Chromatid Exchanges (SCE) which was independent from FA pathway inactivation as the increase was absent in *Fanca*-deficient MEFs [chapter 4]. This increased SCE frequency was not further stimulated by treatment with DSB-inducing agents. This demonstrated that FANCM is not necessary for crossover suppression during DSB repair, but suppressed crossovers in a different context [chapter 4]. In addition, increased SCEs were also observed in DT40 cells mutant for FANCM [29,72]. In DT40 cells increased SCEs are a feature of all FA gene defects, whereas this phenotype was not observed in human or murine FA cells [73]. However, in DT40 cells FANCM and FANCC deficiency was not epistatic in SCE suppression suggesting that FANCM contributes to SCE suppression in DT40 and murine cells in an FA

independent manner [29,45,72].

Thirdly, although in both *Fancf* and *Fancm* mutant mice we observed a statistically significant reduction in overall and tumor free survival [chapters 4 and 5], crossing these mutant mice with the *Apc*^{Min} model revealed a pronounced difference. We found that FANCM deficiency strongly accelerated intestinal tumor development in the ApcMin model, whereas FANCF deficiency had no effect [chapter 6]. This difference in intestinal tumor susceptibility was not associated with an increased frequency of non-sister chromatid exchanges as determined by Mit marker mediated LOH analysis. However, we did find an increased number of chromosomal breaks in untreated primary MEFs unique to *Fancm* deficiency and indicative for the presence of more DSBs. This increased frequency of DSBs in untreated primary *Fancm*-deficient MEF cultures are substrates for HDR with the non-sister chromatid as template leading to LOH and tumor formation in the *Apc*^{Min} model.

Fourthly, it was observed by others that FANCM's ATP dependent capacity to efficiently translocate a branchpoint is critical for normal replication progression and ICL resistance although indispensable for FANCD2 monoubiquitination [31,70]. In addition FANCM was shown to interact with and mediate recruitment of the BTR complex to stalled replication forks. Collectively, these data point to a crucial role for FANCM in normal replication progression independent from the FA pathway.

Concluding remarks on the role of the FA pathway in ICL repair

In the above postulated model for recombination dependent but DSB independent ICL repair it is clear that the FA proteins participate in all the necessary steps. Especially FANCM, FANCD2 and FANCP/SLX4 act as important effectors of a multitude of different steps in ICL repair among others by acting as landing platforms for other proteins. Thus, besides its enzymatic activity as a branchpoint translocase, FANCM may recruit the BRT complex to promote replication fork reversal and reinstallation, and the FA core complex to facilitate ID monoubiquitination and ICL repair. In our model, the FA pathway mediates ICL repair through a TLS-based mutagenic process, thereby preventing gross chromosomal rearrangements [74]. The occurrence of a mutagenic ICL repair mechanism coincides with the observation that base substitutions usually predominate in normal cells, whereas FA cells are hypomutable and instead show deletions [75-77].

Additional experiments in cell free extracts will continue to pinpoint the molecular details of ICL repair and the role of the FA pathway. Especially the sophisticated plasmid based ICL repair system in *Xenopus* egg extract will expand our knowledge on the contribution of the FA pathway in ICL repair. A major point of interest remains the potential additional role of the FA core complex in ICL repair apart from its function as an E3-ubiquitin ligase for FANCD2 and FANCI. Especially as it appears that FANCL alone can monoubiquitinate FANCD2 and confer crosslinker resistance in *Drosophila melanogaster* [78]. Experiments in the past have hinted at this additional function since a monoubiquitinated FANCD2 fusion protein was able to confer ICL

resistance in FANCD2- but not in FANCC-deficient cells, indicating that FANCC is involved in other processes [79]. We now know that the FA core complex has an additional substrate, FANCI, and it would be interesting to test the contribution of the FA core complex and individual members of the FA core complex to restore ICL resistance in the presence of monoubiquitinated FANCD2 and FANCI. Also the identification of new FA genes will add new proteins and insight to the FA pathway.

It is clear that the FA proteins play a pivotal role in crosslink repair, but like FANCM and SLX4 may be involved in other cellular processes as well.

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Addendum A

Summaries, Curriculum Vitae, Publications and Dankwoord

English Summary

Cancer is a disease of the DNA. This means that during the formation of a tumor (tumorigenesis) cells continuously acquire changes (mutations) in their genome . All the hereditary information, collectively referred to as the genome, is in most organisms encoded by DNA. DNA usually has four different building blocks, called bases. These bases (abbreviated as A, C, G and T) are in varying order constricted in a cell and thus read as a genetic code. This code is distributed over a number of individual DNA molecules, called chromosomes. For example humans have 23 pairs of chromosome (one of our mother and one from our father), the mouse 20 and the carp even 52 pairs.

Tumor cells in comparison to normal cells have a large amount of genetic abnormalities or changes in their genetic code. These can be so-called major structural changes when large pieces of DNA are lost, duplicated or pasted together or minor changes when the base sequence is altered. Tumor cells are thus characterized by what we in tumor biology refer to as 'genetic instability'. There are many processes in our daily lives that can lead to genetic changes. For example, exposure to sunlight can cause DNA damage. Fortunately, we have evolved many mechanisms to repair damaged DNA so-called DNA repair mechanisms. UV radiation, for example, glues two T bases together (called a thymine (T) dimmer) making this piece of genetic code unreadable to the cell. A number of specialized enzymes recognize this mistake (lesion in technical jargon) and restore it. The presence of these DNA repair mechanisms in the cell thus prevents genetic instability and hence tumorigenesis. Other mechanisms that ensure genetic stability are aimed at eliminating cells with DNA damage or forcing cells into a state of terminal rest (senescence).

A sad illustration of the importance of DNA repair proteins for genetic instability is the disease Fanconi anemia, the subject of this thesis. Fanconi anemia (FA for short) is a very rare disorder usually diagnosed about 7 years. The disorder is named after Dr. Guido Fanconi (1892-1979), because he was the first physician who described the symptoms. In 1927 he published a case report on a family of five children, where three boys developed anemia between the ages of five and seven, and died. The brothers also had physical defects, such as a small head, skin pigmentation, bleeding, and underdevelopment of the testes. In retrospect, these three brothers are the first documented FA patients and their symptoms are still characteristic of the disease.

In the past FA patients would often die of the consequences of anemia, but improved techniques for bone marrow transplants have led to an increased survival rate for these children. Unfortunately, the disease FA is also characterized by a highly increased risk for cancer. The risk for an FA patient to develop blood tumors is 1000-fold compared to the normal population, 33% of FA patients has developed blood tumors similar at the age of 40. Also the risks for squamous cell carcinomas (SCC) of the head and neck (500-fold increased risk), SCC of the esophagus (1000-fold increased risk), and gynecological cancers are increased.

The symptoms of FA patients appear to be related to a molecular defect in the cells. FA cells (cells from FA patients) have a defect in a specialized DNA repair system, called the FA pathway for repairing DNA crosslinkers. DNA crosslinkers are compounds that nestle between DNA bases and therefore these bases are attached to each other (cross linking). Especially if these crosslinkers are between (inter, ICLs) two DNA strands these compounds are extremely toxic to cells, because the two DNA strands can no longer separate to ensure proper reading of the genetic code. Cells derived from FA patients are therefore very sensitive to exposure with these substances. This sensitivity is exploited in the clinic for diagnosis, since cells display various chromosomal abnormalities seen mainly in FA patients.

In this thesis, I have studied the relationship between a defective FA pathway and its influence on tumorigenesis in mice. In chapter 1, I have provided a comprehensive introduction to the disease Fanconi anemia. Chapter 2 described in detail the current state of affairs regarding the study of Fanconi anemia in mice. By using a molecular biological technique of gene targeting mice can be genetically altered to for example remove a gene. This is called a knock-out mouse. In this thesis I described two mouse models for two different genes related to Fanconi anemia, the gene FANCF (knockout mouse model described in Chapter 5) and the gene FANCM (knockout mouse model described in Chapter 4). Chapter 3 discussed the only patient belonging to the FA-M group, who apart from mutations in FANCM also has mutations in another FA gene, FANCA. Therefore it is questionable whether FANCM can be considered a bona fide FA gene. The mouse model described in Chapter 4 demonstrated that the absence of Fancm in mice indeed led to a similar phenotype as mice lacking a conventional FA gene like Fanca. In Chapter 6 I introduced a new mice cohort which is a cross between the Fancf, Fancm, and a mouse model for colon cancer, the ApcMin the model. I demonstrated that the absence of Fancf had no influence on the development of colon cancer, but the absence of Fancm accelerated this tumor type. I therefore believe that FANCM plays a role as a tumor suppressor independent of its role in the FA pathway, at least in the intestine. In Chapter 7 I introduced my vision on the repair of DNA crosslinkers mediated by the FA proteins, which incorporates new insights gained during my PhD research ,in particular on the role of FANCM, and from the literature and incorporate these into a new model.

Overall this thesis demonstrates the power of studying the contribution of DNA repair proteins on tumorgenesis in mouse models and adds a novel intestinal tumor suppressor, FANCM, to the scientific literature. Future research will uncover whether FANCM also plays a role in human (intestinal) cancers and whether these tumors could be targeted by DNA crosslinking agents.

Nederlandse Samenvatting

Kanker is een ziekte van het DNA. Dit betekent dat tijdens het ontstaan van een tumor (tumorgenese) cellen voortdurend veranderingen (mutaties) in hun genoom vergaren. Met genoom bedoelen wij al de erfelijke informatie die bij de meeste organismen door DNA wordt gecodeerd. DNA kent doorgaans 4 verschillende bouwstenen, basen genoemd. Deze basen (afgekort als A, C, G en T) komen in afwisselende volgorde voor en lezen op deze manier als een genetische code. Deze code is verdeeld over een aantal losse DNA moleculen, chromosomen genoemd. Mensen hebben bijvoorbeeld 23 paar chromosomen (een van onze moeder en een van onze vader), de muis 20 paar en de karper zelfs 52 paar.

Tumorcellen hebben in vergelijking tot normale cellen een grote hoeveelheid genetische afwijkingen oftewel veranderingen in hun genetische code. Dit kunnen grote zogenaamde structurele veranderingen zijn waarbij grote stukken DNA verdwenen, vermenigvuldigd of aan elkaar geplakt zijn oftewel kleine veranderingen waarbij de codering van de basen is veranderd. Tumorcellen worden dus gekenmerkt door wat wij in de tumorbiologie 'genetische instabiliteit' noemen. Nu zijn er in ons dagelijks leven allerlei invloeden die kunnen leiden tot genetische veranderingen. Bijvoorbeeld blootstelling aan zonlicht, hierbij kan ons DNA beschadigd worden. Gelukkig hebben wij in de loop van de evolutie allerlei mechanismen ontwikkeld om deze schade te herstellen, genoemd DNA reparatie mechanismen. Door UV straling worden bijvoorbeeld twee T basen aan elkaar geplakt, dit noemt men een thymine (T) dimeer, waardoor dit stuk genetische code niet meer door de cel afgelezen kan worden. Een aantal gespecialiseerde enzymen herkent deze fout (laesie in vakjargon) en hersteld deze. Het in de cel aanwezig zijn van deze DNA reparatie mechanismen voorkomt dus genetische instabiliteit en daarmee tumorgenese. Andere moleculaire mechanismen die genetische stabiliteit waarborgen zijn gericht op het elimineren van cellen met schade aan het DNA of op het dwingen van cellen in een staat van terminale rust (senescentie).

Een treurige illustratie van het belang van DNA reparatie eiwitten om genetische instabiliteit tegen te gaan en daarmee tumorgenese is de ziekte Fanconi anemia waar dit proefschrift over handelt. Fanconi anemia (afgekort tot FA) is een zeer zeldzame aandoening die meestal gediagnosticeerd wordt rond 7 jaar. De aandoening is vernoemd naar dr. Guido Fanconi (1892-1979), omdat hij de eerste arts was die de symptomen beschreef. Hij publiceerde in 1927 een casus verslag over een gezin van vijf kinderen waarvan drie jongens anemie (bloedarmoede) ontwikkelden in de leeftijd tussen vijf en zeven en stierven. De broers hadden ook lichamelijke afwijkingen, zoals een klein hoofd, huidpigmentatie, bloedingen, en onderontwikkeling van de testes. Achteraf gezien zijn deze drie broers de eerste gedocumenteerde FA-patiënten en hun symptomen zijn nog steeds kenmerkend voor de ziekte.

Vroeger overleden FA-patiënten vaak aan de gevolgen van anemie, maar door de verbeterde technieken voor beenmergtransplantaties overleven deze kinderen nu vaak

tot hun twintigste jaar. Helaas wordt de ziekte FA ook gekenmerkt door een sterk verhoogd risico op kanker. Het risico voor een FA patiënt om bloedtumoren te ontwikkelen is 1000-voudig verhoogd ten opzichte van de gewone populatie; 33% van de FA patiënten ontwikkeld dergelijke tumoren voor het 40ste levensjaar. Ook het risico voor plaveiselcelcarcinomas (SCC) van het hoofd-halsgebied (500-voudig verhoogd risico), SCC van de slokdarm (1000-voudig verhoogd risico), en gynaecologische kankers is sterk verhoogd.

De symptomen van FA patiënten lijken gerelateerd te zijn aan een moleculair defect in de cellen. FA cellen (cellen afkomstig van FA patiënten) hebben namelijk een defect in een gespecialiseerd DNA reparatie systeem, het zogenaamde FA pad om DNA crosslinkers te reparen. DNA crosslinkers zijn stoffen die zich tussen DNA basen nestelen en daardoor deze basen aan elkaar verbinden (crosslinken). Vooral als deze crosslinkers tussen (inter) twee DNA strengen in zitten zijn ze enorm toxisch voor een cel, omdat deze twee DNA strengen dan niet meer gescheiden kunnen worden om de genetische code af te lezen. Cellen afkomstig van FA patiënten zijn dan ook heel erg gevoelig voor blootstelling aan deze stoffen. Bij de diagnose van de ziekte wordt gebruik gemaakt van deze crosslinker gevoeligheid, doordat de cellen na blootstelling allerlei chromosomale afwijkingen laten zien die voornamelijk bij FA patiënten voorkomen.

In dit proefschrift heb ik de relatie tussen een defect FA pad en de invloed hiervan op tumorgenese bestudeerd in muismodellen. In hoofdstuk 1 geef ik een uitgebreide inleiding over de ziekte Fanconi anemie. Hoofdstuk 2 beschrijft in detail de huidige stand van zaken omtrent het bestuderen van Fanconi anemie in muismodellen. Het is met behulp van moleculaire biologische technieken mogelijk om muizen genetisch te bewerken waardoor ze bijvoorbeeld een gen missen. Dit noemt men dan een knock-out muis. In dit proefschrift staan verder twee muismodellen beschreven voor twee verschillende genen die gerelateerd zijn aan de ziekte Fanconi anemia, het gen FANCF (knock-out muismodel beschreven in hoofdstuk 5) en het gen FANCM (knock-out muismodel beschreven in hoofdstuk 4). Hoofdstuk 3 behandelt de enige patiënt behorend tot de FA-M groep, deze bevat behalve mutaties in FANCM ook mutaties in een ander FA gen, FANCA. Hierdoor valt het te betwisten of FANCM een echt FA gen is. Het muismodel beschreven in hoofdstuk 4 beschrijft dat het ontbreken van Fancm in muizen wel degelijk leidt tot een vergelijkbaar phenotype als muizen die Fanca missen, een ander Fanconi anemie gen. In hoofdstuk 6 introduceer ik een nieuwe muizenlijn namelijk een kruising tussen de Fancf en Fancm muismodellen met een muismodel voor darmkanker, het ApcMin model. Ik beschrijf dat het ontbreken van het Fancf gen geen enkele invloed heeft op het ontstaan van darmkanker, maar het ontbreken van Fancm versneld deze tumorsoort. Daarom ben ik van mening dat FANCM een rol speelt als tumor onderdrukker onafhankelijk van zijn rol in het FA pad, althans in de darm. In hoofdstuk 7 introduceer ik mijn visie op het repareren van crosslinkers in het DNA door de FA eiwitten waarbij ik nieuwe inzichten vergaard in dit proefschrift met name op ten opzichte van de rol van FANCM en uit de literatuur incorporeer in een nieuw model.

Samenvattend demonstreert dit proefschrift de kracht van het bestuderen van de contributie van DNA reparatie eiwitten op tumorgenese en voegt een nieuwe (darm) tumor onderdrukker toe aan de wetenschappelijke literatuur. Toekomstig onderzoek moet uitwijzen of ook mensen met (darm) kanker mutaties hebben in FANCM en daardoor wellicht goed behandeld kunnen worden met DNA crosslinkers.

Curriculum Vitae

Sietske Tet Bakker was born in Amsterdam, the Netherlands, on the 24th of August 1979. She attended the Vossius gymnasium high school in Amsterdam from 1991 to 1997. Between 1998 and 2005 she studied at the University of Amsterdam starting with the multidisciplinairy βγ program for first-year students which she completed in chemistry and sociology. She continued her studies in chemistry, specializing in biomedical chemistry. From 2003 to 2004 she joined the group of prof.dr.Piet Borst at the Netherlands Cancer Institute to work on her MSc internship project. From 2005 to 2007, Sietske worked as a PhD student at the Free University in the group of prof. dr.Hans Joenje and dr. Johan de Winter investigating possible synthetic lethal interactions with the Fanconi anemia (FA) pathway. In august 2007 she joined the group of prof.dr. Hein te Riele as a PhD student investigating the significance of the FA pathway in development and treatment of cancer. During her PhD period she studied two FA mouse models: mice disrupted for *Fancf* or *Fancm*. She found that both gene disruptions lead to inactivation of the FA pathway with their corresponding characteristic FA phenotypes. Interestingly, she discovered that *Fancm* disruption in a mouse model for intestinal tumors accelerated tumorigenesis whereas *Fancf* disruption had no effect. This suggested that FANCM plays a unique role in tumor suppression independent of its role in the FA pathway.

Sietske will continue her scientific career as a postdoctoral researcher in the group of dr. Emmanuelle Passegue at the University of San Francisco working on genomic instability in hematopoietic stem cells.

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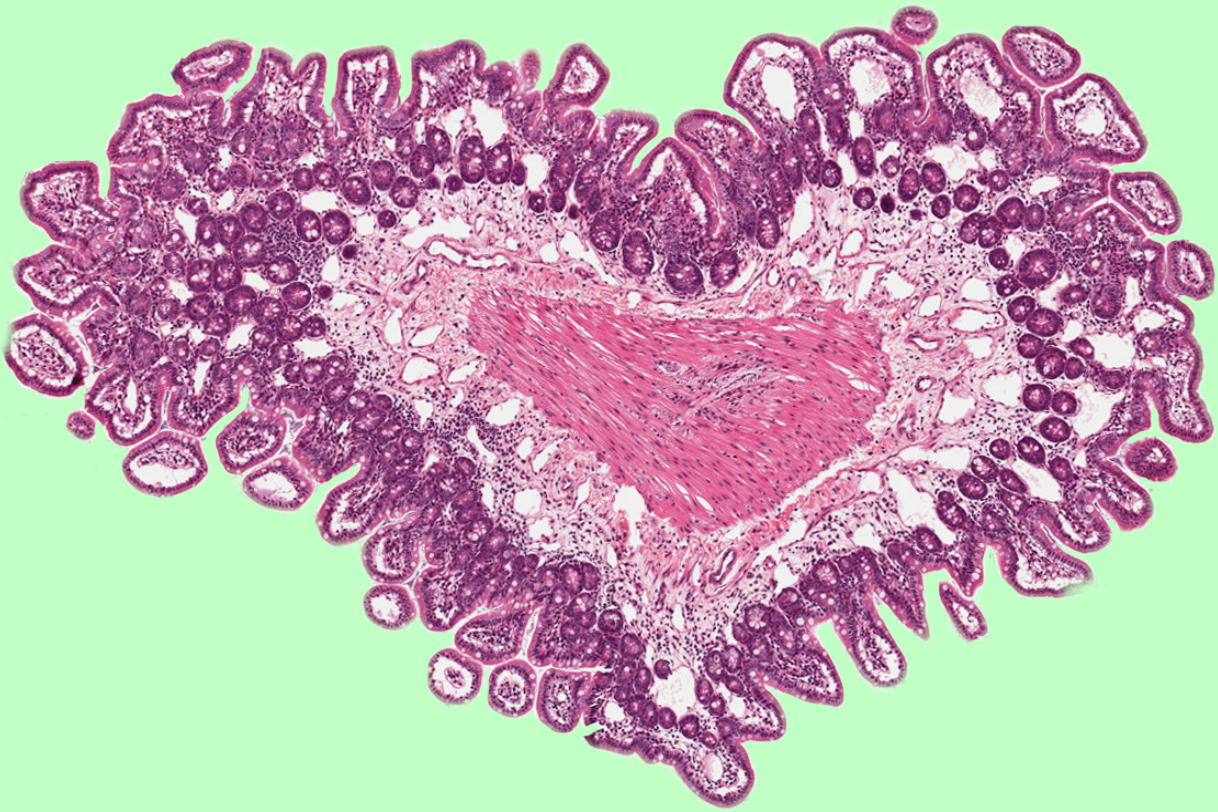
En nu dan eindelijk tijd voor feest,

Lfs Siets



Intestinal Love